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(54) Title: MAMMALIAN DIABETES-MEDIATING PROTEINS

(57) Abstract: Provided are mammalian secreted and non-secreted diabetes mediating proteins, including protective and deleterious diabetes-mediating proteins, as well as polynucleotides encoding same, drug screening methods for identifying a test compound capable of altering the expression of a diabetes-mediating protein, and methods of preventing or ameliorating diabetes by administering a compound capable of altering the expression of a diabetes-mediating protein.

MAMMALIAN DIABETES-MEDIATING PROTEINS

FIELD OF INVENTION

Proteome analysis has allowed for the identification of proteins and their association to diabetes. These proteins, in themselves, either up-regulated or down-regulated, are
5   indicators of diabetes in a patient. The pattern of regulation of a grouping of these proteins also serves as an indicator of diabetes. These proteins can be used as targets for the treatment of diabetes or for treatment itself. The proteins were identified by monitoring IL-1β induced protein changes in diabetes prone mammalian islets of Langerhans.

10   **BACKGROUND OF THE INVENTION**

Type 1 Diabetes Mellitus is a multifactorial polygenetic autoimmune disease, where the insulin producing β-cells are selectively destroyed. The initiating events and precise mechanisms leading to selective β-cell destruction remains unknown. One current hypothesis [3] is that in genetically predisposed individuals the β-cells are influenced by
15   factors from the internal or external environment which can damage the β-cells (e.g. cytokines, virus and chemicals) and then lead to release of β-cell specific proteins. During the destructive process IL-1β is released by macrophages in the islets and the cytotoxic effects of IL-1β on the β-cells results in production of free radicals (e.g. nitric oxide (NO•), super oxide (O_2•) and hydroxyl (OH $^-$) inside the β-cells. Free radicals and NO•
20   are also produced in and secreted from macrophages in the islet infiltrate. The effects of free radicals are attempted scavenged by β-cell protective proteins (e.g. haeme oxygenases and manganese superoxide dismutase (MnSOD)) [25, 26]. A race between protective and destructive mechanisms is initiated, and when the destructive mechanisms exceed the protective mechanisms, the β-cells die [3].

25

Autoimmune insulin-dependent diabetes mellitus (T1DM) is caused by specific destruction of the insulin producing β-cells in the islets of Langerhans in the pancreas. During this process islets are infiltrated with macrophages and lymphocytes, releasing a mixture of cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and
30   interferon-γ (IFN-γ), which is specifically toxic to the β-cells. Cytokines have been demonstrated to induce free radicals such as nitric oxide (NO•), catalyzed by inducible nitric oxide synthase (INOS) and oxygen derived radical.

Development of Type 1 Diabetes Mellitus (T1DM) is characterized by mononuclear cell
35   infiltration in the islets of Langerhans (insulitis) and selective destruction of the insulin

producing β-cells [1, 2]. It is generally accepted that the autoimmune destruction of the β-cells result from interactions between various environmental factors and immune mechanisms in genetically susceptible individuals [3]. The very first events initiating the destructive process has not been described yet. Cytokines, in particular interleukin-1β

5 (IL-1β), are known to be released within the islets in low concentrations by a limited number of nonendocrine cells in sufficient quantities to inhibit and modulate the β-cell function in vitro [4]. In response to low concentrations of IL-1β islets increase insulin release but insulin release is decreased at high concentrations of IL-1β. Furthermore IL-1β influences many important cellular functions such as decreasing DNA synthesis,

10 decreasing protein synthesis and intracellular energy production and induction of apoptosis. Many of these effects are mediated through induction of the inducible NO syntase (iNOS) and its product, the free radical nitric oxide (NO•) [5]. The present investigators hypothesize that the β-cell when exposed to IL-1β initiates a self protective response in competition with a series of deleterious events, and that in β-cells the

15 deleterious prevail [3]. In support of this, overexpression of scavengers of free radicals such as catalase and glutatione peroxidase reduces the deleterious effects of cytokines on β-cells [6].

The present investigators have recently described a similar behavior of Wistar Furth (WF)

20 rat islets exposed to IL-1β in vitro [7]. IL-1β induced significant changes in expression level of 105 proteins in WF islets where both protective proteins e.g. such as galectin-3 and HSP70 are up-regulated and deleterious protein changes e.g. mortalin and lamin A and B1, were identified. In addition proteins involved in mitochondrial energy production were suppressed e.g. adenylate kinase and mitochondrial ATP synthase regulatory

25 subunit B [7].

The BioBreeding diabetes prone (BB-DP) rat spontaneously develops a diabetic syndrome with many characteristics common with human T1DM [8]. Originally the BB-DP rat strain has been breed from a WF rat colony [9]. Strain-dependent variations in β-cell sensitivity to IL-1β effects have been demonstrated in vitro and in vivo [10] [11]. Islets isolated

30 from Brown Norway rats were less sensitive to IL-1β compared to Wistar Furth (WF), Lewis-Scripps (LS) and BB (both diabetes prone (DP) and diabetes resistant (DR)) rats. The BB-DP rat islets produce lower protective stress responses (HSP70) than the diabetes resistant BB rat which might promote enhanced vulnerability and β-cell

35 destruction [12]. The present investigators have previously shown that there is no differ-ence in nitric oxide (NO•) production and 24 hour accumulated insulin release in BB-DP and WF islets in response to exposure to 150pg/ml IL-1β for 24 hours [13]. The higher resistance to IL-1β induced inhibition of β-cell function in vitro and in vivo in BN rat islets

was associated with lower expression of inducible nitric oxide synthase (iNOS) compared to Wistar Kyoto and LS rats [11].

The present investigators previously demonstrated that IL-1β induces reproducible and
5   statistically significant changes in the expression level of 82 protein spots in BB-DP rat islets of Langerhans in vitro out of a total of 1.815 protein spots visualized by 2 dimensional gel electrophoresis. Twenty-two protein spots were up-regulated and 60 protein spots down-regulated [13].

10   Recently, the present investigators have separated approximately 1.900 protein spots according to molecular weight and isoelectric point (pI) by high resolution 2 dimensional gel electrophoresis of WF rat islets of Langerhans [14]. One hundred and five of these protein spots changed expression levels after IL-1β incubation in vitro and the majority of these proteins have now been identified by mass spectrometry. The identified proteins
15   were classified into the following functional groups (in brackets number of different proteins): a) energy transduction and redox potentials (n=14), b) glycolytic enzymes (n=10), c) protein synthesis (n=5), d) chaperones, protein folding and translocation (n=19), e) signal tranduction, regulation, differentiation and apoptosis (n=9) suggesting broad variety of pathways involved in IL-1β toxicity on islets [7].

20   SUMMARY OF THE INVENTION
The present investigators have demonstrated that the combination of 2 dimensional gel electrophoresis and mass spectrometry is a powerful tool in the identification of proteins involved in the cytotoxic effects of IL-β in neonatal BB-DP rat islets. This study demonstrates that 1L-1β exposure of BB-DP and WF rat islets involves essentially the
25   same pathways and results in equal production of NO and accumulated insulin release over 24 hours and the same final result after IL-1β exposure, necrosis and apoptosis although some of the involved proteins are slightly different in the two rat strains.

Moreover, the present investigators have examined the effects of IL-β on BB-DP rat islets
30   in vitro as a simplified model for β-cell destruction in the pathogenesis of T1DM and found that a preponderance of identified changes in protein expression relate to cytokine mediated β-cell destruction and development of T1 DM.

Thus, the present investigators have identified proteins associated with diabetes by
35   detecting the absolute or relative presence of the proteins of tables 1, 2 and 3 in a biological sample. Typically, the biological sample is selected from the group consisting of urine, blood, CSF, saliva, lymphatic fluids, and tissue. Suitably, the tissue is pancreatic islets of Langerhans.

Proteome analysis applied to BB-DP rat islets exposed to IL-1β reveals insight into mechanisms responsible for β-cell destruction at the protein level as well as identifying proteins of relevance in the treatment of diabetes. Furthermore comparison of protein changes identified by proteome analysis in WF and BB-DP rat islets exposed to IL-1β

5   identify proteins and pathways involved in β-cell destruction specific for the diabetes prone BB rat.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates in a first aspect to the novel proteins identified by

10   proteome analysis and mass spectrometry.

A further aspect relates to the use of novel proteins and proteins of table 1 or table 2 or the tables of the mass spectra MWts for the previously unidentified proteins as markers or indicators for diabetes as well as to the use of known proteins whose presence,

15   absence or prevalence has previously not been associated with diabetes. The changes in protein expression and patterns of protein expression are considered to be important markers for diagnosis, prognosis and therapeutic applications and targets.

To identify diabetes marker proteins, IL-1β induced protein changes in BB-DP islets from

20   neonatal BB-DP rats islets were investigated by incubating BB-DP rats islets for 24 hours with or without recombinant human IL-1β. The medium was sampled for nitrite and insulin measurements [13]. Two dimensional gel analysis of the islets was performed and fluorographs of the islets were computer analysed. Protein spots that significantly changed expression level after exposure to IL-1β were cut out of preparatory gels and

25   attempted identified by mass spectrometry.

Proteome analysis, by 2-dimensional gel electrophoresis, mass spectrometry and bio-informatics, offered the opportunity to identify different pathways involved in IL-1β induced 1β-cell destruction. Significantly changed proteins in BB-DP rat islets after IL-1β

30   exposure was obtained.

Similar result were obtained from WF rat islets exposed to IL-1β where 60 protein spots were identified out of 105 significantly changed protein spots. These 60 identifications correspond to 57 different proteins. Comparing the identified proteins in the BB-DP rat

35   with the identified proteins in the WF rat, 13 proteins were identical (NEPHGE (N) 223, 231, 370, 146, 381 (3 spots), IEF (I) 1127, 955, 552, 138 (3 spots), 1136, 8580, 68, 62).

Interestingly, fewer protein spot changed expression levels upon IL-1β exposure in the BB-DP islets (82 spots) compared to WF islets (105 spots). Parts of the difference could be explained by difference in concentration of acrylamide in second dimension gels (Wistar islets were run on 10 and 15% gels whereas BB-DP islets were run on 12.5%

5   acrylamide gels). 33 proteins were changed only in BB-DP islet responses to IL-1β in vitro and 44 proteins "specific" for WF rat islets, although many reflect similar pathways and functions in the two strains studied.

The cytotoxic effect of cytokines on islets is inhibited by inhibitors of protein synthesis

10   indicating that β-cell destruction is an active intracellular process requiring de novo synthesis of proteins. The protein iNOS has previously been reported to change expression level after IL-1β exposure. Heat shock protein 70, 90 and MnSOD others are has been referred to oxidative stress-proteins in connection to their protective response. The proteins of the invention are involved in a variety of different pathways involved in

15   β-cell destruction and may be classified according to the pathway. The proteins of the invention may a) be involved in energy transduction and redox potentials and glycolytic enzymes (14 proteins); b) be involved in purine/pyrimidine synthesis, DNA/RNA synthesis, RNA processing, amino acid metabolism and protein synthesis (7 proteins); c) act as chaperones (5 proteins); d) be involved in differentiation, apoptosis, regulation

20   and signal transduction; e) be involved in cellular defence; f) be involved in cellular structure; g) be involved in hormone/neurotransmitter metabolism; h) or have other functions.

A. Energy transduction and Redox potentials and glycolytic enzymes (14 different

25   _proteins)_
The following proteins of the invention are involved in energy transduction and redox potentials and glycolytic enzymes (14 different proteins):
Malate dehydrogenase precursor;
Aconitate hydratase;

30   Glyceraldehyde-3-phosphate dehydrogenase;
Pyruvate kinase M2 isozyme;
Isocitrate dehydrogenase;
Glucose-6-phosphate dehydrogenase;
ATP synthase;

35   ATP synthase alpha chain;
L-3-hydroxy-CoA dehydrogenase precursor;
NADH dehydrogenase;
Carbonyl reductase;
Vacuolar ATP synthase subunit B;

Alcohol dehydrogenase; and

Creatine kinase B chain was identified together with cytokeratine 8 polypeptide

are each diabetes marker proteins of the present invention.

5   IL-1β induces NO production in the 1β-cells which in turn nitrosylates the Fe-S complex

in enzymes [31] and inactivates mitochondrial aconitase [32] whereby the oxidation of

glucose in the Krebs Cycle is inhibited which results in decreased ATP production.

The malate dehydrogenase precursor has been found down-regulated in three spots,

10   Aconitate hydratase, glyceralaldehyde-3-phosphate dehydrogenase, pyruvate kinase M2

isozyme and isocitrate dehydrogenase precursor are all down-regulated and involved in

energy production in the Krebs Cycle.

Glucose-6-phosphate dehydrogenase, the first step in the pentose phosphate pathway is

15   up-regulated, which could be to compensate for the decreased energy production

through the Krebs Cycle.

Furthermore several proteins involved in energy transduction and redox potentials are

down-regulated in response to IL-1β exposure. The proteins are H+ transporting ATP

20   synthase, ATP synthase alpha chain, L-3-hydroxy-CoA dehydrogenase precursor, NADH

dehydrogenase, carbonyl reductase, vacuolar ATP synthase subunit B and alcohol

dehydrogenase.

Taken together this strongly suggest that energy production in islets is inhibited by IL-1β

25   in BB-DP rat islets.

Creatine kinase B chain was identified together with cytokeratine 8 polypeptide in an up-

regulated spot. Creatine kinase was found down-regulated in WF islets [7] indicating that

creatine kinase could also be involved here.

30

B. Purine/pyrimidine synthesis, DNA/RNA synthesis, RNA processing, amino acid

metabolism and protein synthesis (8 proteins)

The following proteins of the invention are involved in purine/pyrimidine synthesis,

DNA/RNA synthesis, RNA processing, or amino acid metabolism and protein synthesis (8

35   different proteins:

5-aminoimidazole-4-carboxamide ribonucleotide formyltranferase;

UMP-CMP kinase;

Adenine phosphoribosyltransferase;

Heterogeneous nuclear ribonucleoprotein A2/1311;

Heterogeneous nuclear ribonucleoprotein M (M4 protein deletion mutant);

Aspartate aminotransferase (cytosolic);

Aspartate aminotransferase (mitochondrial); and

Ribosomal protein L11

5   are each further diabetes marker proteins of the present invention.

Three of the identified proteins, 5-aminoimidazole-4-carboxamide ribonucleotide

formyltranferase, UMP-CMP kinase and adenine phosphoribosyltransferase, involved in

the synthesis of purine and pyrimidine, were down-regulated after exposure to IL-1β.

10

Heterogeneous nuclear ribonucleoprotein A2/1311 and M4 protein deletion mutant

(heterogeneous nuclear ribonucleoprotein M) are both involved in processing of preRNA

[33, 34] and were both down-regulated too. Aspartate aminotransferase, both the

cytosolic and the mitochondrial form, involved in amino acid metabolism were down-

15   regulated after exposure to IL-1β. The down-regulation of all these proteins support that

the β-cells focus their efforts in protecting themselves against the deleterious effects of

IL-1β and down-regulate functions that are not involved in protection.

C. Chaperones (7 different proteins)

20   Endoplasmin;

GRP 78 [39] 78kDa glucose regulated protein (GRP78);

Protein disulfide isomerase;

Probable disulfide isomerase P5;

Calreticulin;

25   Endoplasmic reticulum protein ERP 29; and

HSP70

are each further diabetes marker proteins of the present invention.

Endoplasmin, one of two proteins in a down-regulated spot, belongs to the HSP 90 family

30 '   which is involved in protein folding [38] and has been shown to have serine kinase

activity which is enhanced by association with 78kDa glucose regulated protein (GRP78)

[39].

GRP78, was found in three spots, one up-regulated, one down-regulated and together

35   with endoplasmin in one down-regulated spot (demonstrating complex differential

regulation of post-translational modifications). It is a member of the HSP 70 family

involved in the folding and assembly of proteins in the endoplasmatic reticulum [40].

Endoplasmic reticulum protein ERP 29 is a member of the ER protein-processing

machinery and is involved in protein secretory events. IL-1β exposure down-regulates this protein.

Protein disulfide isomerase, involved in molecular chaperone of glycoprotein biosynthesis
5   [41] is down-regulated by IL-1β together with probable disulfide isomerase P5. Both are involved in the formation and rearrangement of disulfide bonds in proteins. Interestingly, all these proteins are able to bind calcium like calreticulin [42] and may also be involved in signal transduction.

10   Up-regulated HSP70 expression has been demonstrated to diminish the inhibitory effect of IL-1β on islet insulin secretion and NO-induced mitochondrial impairment [43, 44]. In the diabetes prone BB rat, insufficient HSP70 expression in islets has been correlated to sensitivity to NO• and oxygen radical toxicity [12].

15   _D. Differentiation, apoptosis, regulation and signal transduction (7 different proteins)_
     Galectin-3;
     Eukaryotic initiation factor 4A;
     Toad 64;
     Secretagogin;
20   Calreticulin;
     Craniofacial development protein 1; and
     The Voltage dependent anion channel protein
     are each further diabetes marker proteins of the present invention.

25   IL-1β induces cell death through different pathways, among them gene controlled apoptosis [45-47]. Galectin-3, a protein involved in cell survival by inhibiting apoptosis [48 - 50] is up-regulated and present in 2 spots.

     Eukaryotic initiation factor 4A, like NUK 34, is involved in transcriptional/translational
30   regulation [51] is up-regulated indicating specific activation of protein synthesis.

     Toad 64, a protein up-regulated over the course of neurogenesis and playing a role in signal transduction processes involved in axon growing [52] is up-regulated after IL-1β exposure.
35

     The calcium binding protein, calreticulin, found up-regulated in two spots, can act as an important modulator of the regulation of gene transcription by nuclear hormone receptors (glucocorticoid and androgen receptors) [55] [56]. Taken together the changes

in expression level of these proteins indicate that proteins potentially involved in apoptosis is changed by IL-1β exposure.

Another calcium binding protein, secretagogin, specific for neuroendocrine cells and related to calbindin D-28k, is involved in cell growth and maturation [53] and is down-regulated. Calbindin D-28k has been shown to be involved in protection from cytokine induced apoptosis in 13-β cells [54].

Voltage dependent anion channel is identified in a down-regulated spot together with L-3-hydroxyacyl-CoA dehydrogenase precursor. The Voltage dependent anion channel protein is able to form small pores in the outer mitochondrial membrane allowing movements of adenine nucleotides. Bcl2 proteins binds to the channel in order to regulate the mitochondrial membrane potential and release of cytochrome c during apoptosis [57].

E. Cellular defence (2 proteins)
Catalase; and
Glutathione synthetase
are each further diabetes marker proteins of the present invention.

Free radicals (e.g. nitric oxide and hydrogen peroxide) are proposed to play a central role in the destruction of β-cells [3]. Catalase, which serves to protect cells from the toxic effects of hydrogen peroxide, is up-regulated in response to IL-1β exposure.

Overexpression of catalase, super oxide dismutase and glutathione peroxidase in Rinm5F cells protect against the toxicity of NO donors [58]. The increased expression of catalase in the BB-DP islets apparently is not sufficient to protect against the IL-1β induced free radicals. Hereditary catalase deficiencies are reported to increase the risk for T1 DM in a Hungarian population [59].

Glutathione synthetase is important for a variety of biologic functions including protection of cells from oxidative damage by free radicals and detoxifications [58, 60 and 61] and is here reduced to one eighth of the normal concentration (down-regulated) suggesting a reduced potential for cellular defence against oxygen-derived free radicals.

F. Cellular structure (3 proteins)
Tubulin beta-5 chain;
Cytokeratin 8; and
Keratin 2a

are each further diabetes marker proteins of the present invention.

Tubulin beta-5 chain, the major constituent of microtubules were present in 4 spots,
possibly 4 different modifications, all down-regulated probably due to lower mitotic rate

5   upon IL-1β exposure. Cytokeratin 8 was present in the same up-regulated spot as
creatine kinase-B. Cytokeratin 8 has been shown to play an essential role in regulation of
growth and differentiation in the exocrine pancreas [62]. Furthermore keratin 2a is found
together with tubulin beta-5 chain in a down-regulated spot.

10   _G. Hormone/neurotransmitter metabolism (5 different proteins)_
Membrane associated progesterone component (25-Dx);
Amyloid beta-peptide binding protein;
Neuroendocrine convertase 1;
Neuroendocrine convertase 2; and

15   Beta-alanine oxoglutarate aminotransferase (GABA-transaminase)
are each further diabetes marker proteins of the present invention.

Membrane associated progesterone component (25-Dx) is a receptor for progesterone
which is massively down-regulated after IL-1β exposure. 25-Dx has 71 % sequence

20   homology with the transmembrane domain of the precursor for the IL-6 receptor and a
conserved consensus sequence found in the cytokine/growth factor prolactin receptor
superfamily [63].

Amyloid beta-peptide binding protein is involved in androgen metabolism and has been

25   postulated to be involved in apoptosis and amyloid toxicity [64]. Here, it is down-
regulated by 1L-1β.

Beta-alanine oxoglutarate aminotransferase (GABA-transaminase) is responsible for the
catabolism of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and is

30   down-regulated in IL-β exposed islets. It probably has a different function in the β-cells.

H. Other functions (2 proteins)
5-aminolevulinate synthase precursor; and
the cytomegalovirus protein immediate-early protein 1

35   are each further diabetes marker proteins of the present invention

5-aminolevulinate synthase precursor is a rate limiting nuclear-encoded mitochondrial
enzyme in the heme biosynthetic pathway which serve as part of several heme
containing proteins such as hemoglobin and catalase. IL-1β down-regulates the

expression of 5-aminolevulinate synthase precursor and might then increase the β-cell susceptibility to IL-1β due to reduced levels of one of the substrates for catalase production.

5   Interestingly the cytomegalovirus protein immediate-early protein 1 has been identified in the BB-DP islets as down-regulated. This protein is able to transactivate heterologous promoters [65] in rat cytomegalovirus. The protein may come from a cytomegalovirus infection or may be a homologous rat protein.

10 Exposure of BB-DP rat islets of Langerhans to 150 pg/ml of human IL-1β for 24 hours induces significant changes in expression levels of 82 proteins. Positive protein identification has been obtained for most spots by mass spectrometry, which at present is the most powerful method and technique of choice to identify proteins from high resolution two-dimensional gels [66]. It is understood by the person skilled in the art

15 that the effect of human IL-1β on rat islets is the same effect as that anticipated on human islets.

In Table 1, proteins common for BB-DP and WF are marked with #. From Table 1 it is evident that proteins involved in chaperoning, protein folding and translocation and

20 proteins involved in energy transduction and redox potentials are common pathways affected in the two strains. For both groups most of the proteins are down regulated suggesting that similar mechanisms are affected by IL-1β exposure in the two rat strains. Another pattern is seen for the glycolytic enzymes. Here the outcome of the changes in expression levels of proteins, a lower energy production, although there is only overlap

25 in 2 out of 8 proteins in the BB-DP rat [7]. This suggests that IL-1β affects the glycolytic enzymes differently in the two rat strains with the same final result, a lower energy production.

Two proteins involved in cellular defense, catalase and glutathione synthetase are both

30 changed in expression levels by IL-1β in BB-DP rats but not in WF rat islets. The increased expression of catalase protects cells against the free radical hydrogen peroxide indicating that free radicals are present in the BB-DP islets after IL-1β exposure and that the islets are trying to protect themselves against the free radicals. The previously described strain differences in rat islets regarding IL-1β sensitivity and NO• production

35 after IL-1β exposure might be explained by different abilities to mount a free radical defense [10].

The other protein involved in cellular defense, glutathione synthetase involved in energy requiring synthesis (ATP is one of the substrates) of the free radical scavenger

glutathione is decreased by more then seven fold. This indicates that the glutathione detoxifying pathway is impaired by IL-1β in BB-DP rats.

In the functional studies of the islets in vitro, IL-1β increased the NO• production over a 24 hour period, but iNOS was not identified as one of the up-regulated proteins. This could be explained by absence of IL-β in the 4 hours labeling period after the IL-1β incubation or that the difference in expression level does not fulfil our criteria for significant difference or is not present in the gels. The same was seen in WF islets incubated with IL-1β [7]. Increased mRNA levels for iNOS has been found in prediabetic NOD mice [67].

Taken together, the comparison of IL-1β exposed neonatal BB-DP islets and WF islets has revealed that IL-β induce significant changes in the expression level of, respectively, 82 and 105 protein spots. There is an overlap in 13 of the identified proteins, and involvement of the same pathways, suggesting that different proteins are expressed upon IL-1β exposure in BB-DP and WF rat islets although there is no difference in NO• and insulin and that the final result, apoptosis and β-cell destruction is the same.

Some proteins are seen in more than one spot, e.g. calreticulin and GRP 78, which indicate that the protein is present in more than one form presumably due to post-translational modifications. Post-translational modifications, such as phosphorylations, methylations or glycosylations are important for the function or activation of the protein. Proteins with known genes and chromosomal localisation close to known T1 DM loci in the human genome might be of particular interest and might identify primary changes.

A first aspect of the invention relates to marker proteins. The marker proteins are selected from the group consisting of:

a)   a protein of table 1, a protein of table 2, a protein of table 3, a protein of figure 1 (such as a protein marked with a number) and a protein having a spectrum as shown in any one of the figures 5-21; and

b)   a protein with at least 80% sequence homology with a protein in a).

Also, the marker protein can be selected from the group consisting of:

a)   one or more proteins present in a significantly lower or significantly higher amount on a polyacrylamide gel of proteins from said biological sample in relation to a control;

b)   one or more proteins present on a polyacrylamide gel of proteins from said biological sample and absent on polyacrylamide gel of proteins of a control; and

c)   one or more proteins absent on a polyacrylamide gel of proteins from said
biological sample and present on polyacrylamide gel of proteins of a control.

The meaning of the term "control" is evident for a person skilled in the art of proteomics.
5   Preferably, the control is an IOD% value of a spot (having the same position) from a gel
of proteins from a sample originating from a human who is not predisposed for or having
diabetes.

The term "marker protein" is meant to encompass the above mentioned proteins, as well
10   as similar proteins from other species of mammals. The human forms of the proteins are
of particular interest. For example, it is suggested that the human forms of the rat
proteins can be used as markers for diabetes in human beings. Thus, the term "marker
protein" encompasses the human form of the identified proteins in table 1 and the not
yet identified proteins in table 2, as well as the proteins being up- or down-regulated in a
15   mammalian tissue sample (e.g. human) as described elsewhere. Examples of the marker
proteins of the invention are given below.

An other aspect of the invention relates a method for diagnosing or for determining the
predisposition of diabetes in a mammal, e.g. in a human, the method comprising
20   determining the presence, absence or level of expression of at least one marker protein
in a biological sample from said mammal. The biological sample can be selected from the
group consisting of urine, blood, saliva, lymphatic fluids, and tissue, presently pancreatic
tissue is preferred.

25   A presently preferred method comprises establishing the increased expression of at least
one marker protein (an up-regulated marker protein) selected from the group consisting
of:
a)   a protein which is defined in table 1 and/or 2, and marked as up-regulated; and
b)   a protein which is a modification or a derivative of a protein in a), so as to have
30   at least 80% sequence homology with a protein in a),
but of course the invention also relates to a method establishing the decreased
expression of at least one down-regulated marker protein.

In another embodiment, the invention relates to a method for determining the
35   predisposition for diabetes in a human, the method comprising:
a)   · determining the increased expression of at least one marker protein in a
biological sample originating from the human, said marker protein being selected from
the group consisting of: i) an up-regulated protein defined in table 1, 2 and/or 3, and ii)

a protein which is a modification or a derivative of a protein in i), so as to have at least 80% sequence homology with a protein in i);

b)   determining the decreased expression in a biological sample from the human of at least one marker protein, said marker protein being selected from the group

5   consisting of: i) a down-regulated protein defined in table 1, 2 and/or 3 and ii) a protein which is a modification or a derivative of a protein in i), so as to have at least 80% sequence homology with a protein in i) ; or

c)   combinations of the determinations in a) and b).

10   Thus, the determination of whether a protein is up-regulated or down-regulated serves as useful indicators of diabetes susceptibility. The pattern of up and down regulation may also serve as an indicator. That is to say that the level of expression of more than one protein is established and the pattern of expression of a grouping of proteins is used as an indicator.

15

In a suitably embodiment, at least one marker protein is selected from the group consisting of one or more proteins present in a significantly lower or significantly higher amount on a polyacrylamide gel of proteins from said biological sample in relation to a control, one or more proteins present on a polyacrylamide gel of proteins from said

20   biological sample and absent on polyacrylamide gel of proteins of a control, one or more proteins absent on a polyacrylamide gel of proteins from said biological sample and present on polyacrylamide gel of proteins of a control.

In another embodiment, the invention relates to a method of treating diabetes, or

25   preventing or delaying the onset or of diabetes, in a mammal, e.g. in a human, comprising altering the expressing of a least one marker protein.

The method preferably comprises administering a compound selected from the group consisting of:

30   a)   a protein which is defined in table 1, 2 and/or 3;

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3;

c)   a protein having the same function as a protein in a) and/or b);

d)   a nucleotide sequence coding for a protein in a), b) or c);

35   e)   an antibody for a protein of a), b) or c);

f)   a nucleic acid fragment capable of binding to a protein of a) b) or c); and

g)   a compound capable of binding to a protein of a), b) or c)

to said human.

Also, the invention relates to the use of such a compound for the manufacture of a medicament for the treatment or prophylaxis of diabetes. The term "diabetes" comprises diseases associated with diabetes, especially with type 1 diabetes mellitus.

5 With regard to a method of treating diabetes, a single protein may be targeted for therapy or a grouping of proteins may be targeted. The level of expression of these targeted proteins may be altered or the proteins themselves may be interfered with in order to alter their activity. Thus, an interesting embodiment of a method of treating diabetes in a human comprises altering the expressing of a marker protein.

10

In another embodiment, the invention relates to a method of determining the likelihood of an agent having a therapeutic effect in the treatment of diabetes comprising determining the relative level of expression of one or more marker proteins before and after exposing a test model to said agent.

15

Also, the invention relates to a method of determining the effect of a compound in the treatment of diabetes comprising determining the level of expression of proteins of one or more marker proteins.

20 In a further embodiment, the invention relates to a method of determining the level of effect of a compound used in the treatment of diabetes comprising determining the level of expression of one or more marker proteins before and after exposing a test model to said agent.

25 Also, the invention relates to a method of determining the nature or cause of diabetes in a mammal, e.g. in a human, having or susceptible to said disease comprising establishing the level of expression of a at least one marker protein in relation to a model.

30 An other embodiment of the invention relates to a nucleic acid fragment comprising a nucleotide sequence which codes for a marker protein, or to a nucleic acid fragment which hybridizes with a such a nucleic acid fragment or a part thereof or with its complementary strand.

35 The invention relates to the use of the above mentioned nucleic acid fragments for detecting the presence of a marker protein.

In yet another embodiment, the invention relates to an antibody able to bind to a marker protein. Such an antibody can be a polyclonal antibody or a monoclonal antibody. The

invention also relates to the use of such an antibody for detecting the presence of a marker protein.

In a further embodiment, the invention relates to a test kit for diagnosing diabetes or a
5   genetic predisposition for diabetes in a mammal, e.g. in a human, comprising:

a)   a binding means which specifically binds to at least one marker protein; or which binding means is an antibody for at least one said marker protein, a nucleic acid fragment capable of binding to at least one said marker protein, or a compound capable of binding to at least one said marker protein;

10   b)   a means for detecting binding, if any, or the level of binding, of the binding means to at least one of the marker proteins or to at least one of the nucleic acid fragments, and

c)   a means for correlating whether binding, if any, or the level of binding, to said binding means is indicative of the individual mammal having a significantly higher like-
15   lihood of having diabetes or a genetic predisposition for having diabetes.

In an other embodiment, the invention relates to a method for determining the effect of a substance, the method comprising using a mammal, e.g. a human, which has been established to be an individual having a high likelihood of having diabetes or a genetic
20   predisposition for having diabetes by use of a method of the invention, the method comprising administering the substance to the individual and determining the effect of the substance.

The present investigators anticipate that a method of determining the nature or cause of
25   diabetes in a human having or susceptible to said disease comprising establishing the level of expression of a protein of Table 1 in relation to a model serves for understanding the disease and potential therapies.

In the testing of compounds, knowledge about the activity or target of an agent is useful
30   for understanding the therapeutic activity of said agent and may assist in improving the desired therapy. The developments of the present investigators allows for a method of determining the effect of a compound in the treatment of diabetes comprising determining the level of expression of one or more marker proteins and to a method of determining the level of effect or level of activity of a compound used in the treatment of
35   diabetes comprising determining the level of expression of one or more marker proteins before and after exposing a test model to said agent.

A very important embodiment of the invention relates to a pharmaceutical composition which comprises:

a)   a substance which is capable of regulating the expression of a nucleic acid frag-
ment coding for at least part of a marker protein;

b)   a marker protein;

c)   a derivative, homologue or mimic of a marker protein;

d)   an antibody for a marker protein;

e)   a nucleic acid fragment capable of binding to a marker protein; and/or

f)   a compound capable of binding to a marker protein.

The pharmaceutical composition can be used as a medicament, such as for treatment or
prophylaxis of diabetes.

It should be noted that the detection of any combination of more than one of the
markers would be expected to make the analysis an even more reliable indicator for the
disease related to diabetes. Thus, a method for diagnosing or determining the
predisposition of at least one disease related to diabetes comprising determining the
presence, activity, concentration and/or level of expression of a combination of two
markers would be preferred and three or more markers (e.g. at least 4, 5, 6 or 7
markers) would be strongly preferred. It is analogously suggested that treatment with
more than one compound (e.g. at least 2, 3, 4, 5, 6 or 7 compounds) according to the
invention (e.g. more than one compound chosen from the group consisting of: a
polypeptide, a nucleic acid fragment or an antibody according to the invention), said
compounds combined being able to affect the level of more than one marker protein,
would make the treatment of the disease even more efficient.

The term "polypeptide" in the present invention should have its usual meaning. That is
an amino acid chain of any length, including a full-length protein, oligopeptides, short
peptides and fragments thereof, wherein the amino acid residues are linked by covalent
peptide bonds. The terms "polypeptide", "peptide", "amino acid sequence" and "protein"
are used interchangeably.

The protein may be chemically or biochemically modified by being phosphorylated,
methylated, sulphylated, glycosylated or by the addition of any form of lipid or fatty acid,
ubiquitin or any other large side groups or by containing additional amino acids or any
other forms of modification (of which there are over 200 known). These modifications
occur at specific sites on the protein and a particular modification at one site can have
different effects as the same modification at a different site on the same protein. They
can be reversible in the cell where they are used for example to turn on and off enzymes
and so the proteins can exist in a variety of forms – each with an associated activity level

for each of the proteins functions. Furthermore the polypeptide may be cleaved e.g. by processing at its N- or C-termini to remove signal peptides or be spliced to remove an internal sequence. Examples of many of these can be found in the protein databases like EXPASY and there exist an ever growing range of tools to predict these modifications and

5   their function (see http://www.expasy.ch/). Since it is estimated that each protein in man is modified on average 10 times, it is expected that the majority of the proteins identified here are modified in some way or another. Their apparent isoelectric point and molecular weight has thus been given in tables 1, 2 and 3 so that they can be compared to the theoretical values to indicate what effects the modification has had on the protein.

10

Each polypeptide may thus be characterized by specific amino acids and be encoded by specific nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant or synthetic methods wherein such polypeptide sequences have been modified by substitution, insertion, addition or deletion of

15   one or more amino acid residues in the recombinant polypeptide and still be immunogenic in any of the biological assays described herein. Substitutions are preferably "conservative". Conservative substitutions are known to a person skilled in the art. Preferably, amino acids belonging to the same grouping (non-polar (G, A, P, I, L and V), polar-uncharged (C, S, T, M, N and Q), polar-charged (D, E, K and R) and

20   aromatic (H, F, W and Y)). Within these groups, amino acids may be substituted for each other, but other substitutions are of course possible.

Each polypeptide is encoded by a specific nucleic acid sequence. It will be understood that such sequences include analogues and variants hereof wherein such nucleic acid

25   sequences have been modified by substitution, insertion, addition or deletion of one or more nucleic acid residues (including the insertion of one or more introns (small or large)). Substitutions are preferably silent substitutions in the codon usage, which will not lead to any change in the amino acid sequence, but may be introduced to enhance the expression of the protein.

30

In the present context the term "substantially pure polypeptide" means a polypeptide preparation which contains at most 5% by weight of other polypeptide material with which it is natively associated (lower percentages of other polypeptide material are preferred, e.g. at most 4%, at most 3%, at most 2%, at most 1%, and at most ½%). It

35   is preferred that the substantially pure polypeptide is at least 96% pure, i.e. that the polypeptide constitutes at least 96% by weight of total polypeptide material present in the preparation, and higher percentages are preferred, such as at least 97%, at least 98%, at least 99%, at least 99,25%, at least 99,5%, and at least 99,75%. It is especially preferred that the polypeptide fragment is in "essentially pure form", i.e. that

the polypeptide fragment is essentially free of any other protein with which it is natively associated, *i.e.* free of any other protein from a mammal. This can be accomplished by preparing the polypeptide of the invention by means of recombinant methods in a host cell as known to a person skilled in the art, or by synthesizing the polypeptide fragment

5 by the well-known methods of solid or liquid phase peptide synthesis, e.g. by the method described by Merrifield (Merrifield, R. B. Fed. Proc. Am. Soc. Ex. Biol. 21: 412, 1962 and J. Am. Chem. Soc. 85: 2149, 1963) or variations thereof, or by means of recovery from electrophoretic gels.

10 The term "protein" also encompasses derivatives, analogues and mimetics of the above mentioned polypeptides. Such a derivative, analogue and mimetic preferably have the same activity, e.g. the same kind of enzymatic activity, as the polypeptide which it is derived from. The derivative, analogue or mimetic can have a lower level activity, the same level or preferably, a higher level of activity than the parent polypeptide.

15

The term "a least one" (e.g. at least one compound or at least one marker protein) encompasses the integers 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 etc. It should be understood that a single marker protein can be used, but it can be advantageous to use more than one marker protein in methods of the invention. That is

20 to say that the level of expression of more than one protein is established and the pattern of expression of a grouping of proteins is used as an indicator. Obviously the reliability of identification increases as the number in the group increase.

A "peptide mimetic" is a molecule that mimics the biological activity of a peptide but is

25 no longer peptidic in chemical nature. By strict definition, a peptidomimetic is a molecule that no longer contains any peptide bonds (that is, amide bonds between amino acids). However, the term peptide mimetic is sometimes used to describe molecules that are no longer completely peptidic in nature, such as pseudo-peptides, semi-peptides and pep-toids. Whether completely or partially non-peptide, peptidomimetics according to this in-

30 vention provide a spatial arrangement of reactive chemical moieties that closely resem-bles the three-dimensional arrangement of active groups in the peptide on which the peptidomimetic is based. As a result of this similar active-site geometry, the peptidomimetic has effects on biological systems, which are similar to the biological activity of the peptide. The present invention encompasses peptidomimetic compositions

35 which are analogs that mimic the activity of biologically active peptides according to the invention, i.e. the peptidomimetics can be used for treatment of diabetes related diseases. The peptidomimetic of this invention are preferably substantially similar in both three-dimensional shape and biological activity to the peptides or active sites of such as set forth above.

Alternatively, the mimetic can be an 'antimimetic'. In other words, a molecule that can fit into and block the active site of the protein, or bind to binding sites or sites of interaction with other biological molecules and so interfere with the function of the protein. Most

5   current drugs are of this type. Such antimimetics that are capable of interacting with the polypeptides of the invention are encompassed by the present invention.

There are clear advantages for using a mimetic of a given peptide rather than the peptide itself, because peptides commonly exhibit two undesirable properties: (1) poor

10   bioavailability; and (2) short duration of action. Peptide mimetics offer an obvious route around these two major obstacles, since the molecules concerned are small enough to be both orally active and have a long duration of action. There are also considerable cost savings and improved patient compliance associated with peptide mimetics, since they can be administered orally compared with parenteral or transmucosal administration for

15   peptides. Furthermore, peptide mimetics are much cheaper to produce than peptides. Finally, there are problems associated with stability, storage and immunoreactivity for peptides that are not experienced with peptide mimetics.

Thus peptides described above have utility in the development of such small chemical

20   compounds with similar biological activities and therefore with similar therapeutic utilities. The techniques of developing peptidomimetics are conventional. Thus, peptide bonds can be replaced by non-peptide bonds that allow the peptidomimetic to adopt a similar structure, and therefore biological activity, to the original peptide. Further modifications can also be made by replacing chemical groups of the amino acids with

25   other chemical groups of similar structure. The development of peptidomimetics can be aided by determining the tertiary structure of the original peptide by NMR spectroscopy, crystallography and/or computer-aided molecular modelling. These techniques aid in the development of novel compositions of higher potency and/or greater bioavailability and/or greater stability than the original peptide [Dean (1994), BioEssays, 16: 683-687;

30   Cohen and Shatzmiller (1993), J. Mol. Graph. 11: 166-173; Wiley and Rich (1993), Med. Res. Rev., 13: 327-384; Moore (1994), Trends Pharmacol. Sci., 15: 124-129; Hruby (1993), Biopolymers, 33: 1073-1082; Bugg et al. (1993), Sci. Am., 269: 92-98, all incorporated herein by reference]. Once a potential peptidomimetic compound is identified, it may be synthesized and assayed using the diagnostic assay described herein

35   or an appropriate disease suppressor assay [see, Finlay et al. (1983), Cell, 57: 1083-1093 and Fujiwara et al. (1993), Cancer Res., 53: 4129-4133, both incorporated herein by reference], to assess its activity.

Thus, through use of the methods described above, the present invention provides com-

pounds exhibiting enhanced therapeutic activity in comparison to the polypeptides described above. The peptidomimetic compounds obtainable by the above methods, having the biological activity of the above named peptides and similar three dimensional structure, are encompassed by this invention. It will be readily apparent to one skilled in the

5   art that a peptidomimetic can be generated from any of the modified peptides described previously or from a peptide bearing more than one of the modifications described previously. It will furthermore be apparent that the peptidomimetics of this invention can be further used for the development of even more potent non-peptidic compounds, in addition to their utility as therapeutic compounds.

10

By the terms "nucleic acid fragment" and "nucleic acid sequence" and the like are understood any nucleic acid molecule including DNA, RNA, LNA (locked nucleic acids), PNA, RNA, dsRNA and RNA-DNA-hybrids. Also included are nucleic acid molecules comprising non-naturally occurring nucleosides. The term includes nucleic acid molecules

15   of any length, e.g. from 10 to 10000 nucleotides, depending on the use. When the nucleic acid molecule is for use as a pharmaceutical, e.g. in DNA therapy, or for use in a method for producing a polypeptide according to the invention, a molecule encoding at least a part of the polypeptide is preferably used, having a length from about 18 to about 1000 nucleotides, the molecule being optionally inserted into a vector. When the nucleic

20   acid molecule is used as a probe, as a primer or in antisense therapy, a molecule having a length of 10-100 is preferably used. According to the invention, other molecule lengths can be used, for instance a molecule having at least 12, 15, 21, 24, 27, 30, 33, 36, 39, 42, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500 or 1000 nucleotides (or nucleotide derivatives), or a molecule having at most 10000, 5000, 4000, 3000, 2000, 1000, 700,

25   500, 400, 300, 200, 100, 50, 40, 30 or 20 nucleotides (or nucleotide derivatives). It should be understood that these numbers can be freely combined to produce ranges.

The term "stringent" when used in conjunction with hybridization conditions is as defined in the art, i.e. the hybridization is performed at a temperature not more than 15-20°C

30   under the melting point (Tm) of the nucleic acid fragment, cf. Sambrook *et al* Molecular Cloning; A laboratory manual, Cold Spring Harbor Laboratories, NY, 1989, pages 11.45-11.49. Preferably, the conditions are "highly stringent", i.e. 5-10°C under the melting point (Tm). In the present invention, the hybridisation conditions are preferably stringent.

35

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations thereof such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The term "sequence identity" (or "sequence homology") indicates a quantitative measure of the degree of homology between two amino acid sequences of equal length or between two nucleotide sequences of equal length. If the two sequences to be compared

5   are not of equal length, they must be aligned to best possible fit possible with the insertion of gaps or alternatively truncation at the ends of the protein sequences. The se-

quence identity can be calculated as $\frac{(N_{ref}-N_{dif})100}{N_{ref}}$, wherein N_{dif} is the total number of non-

identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a

10 sequence identity of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC ($N_{dif}=2$ and $N_{ref}=8$). Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988) PNAS USA 85:2444-

15 2448)(www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In one aspect of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al Nucleic Acids Res 1994 22:4673-4680, available at http://www2.ebi.ac.uk/clustalw/. Alternatively, the degree of homology between two nucleic acid sequences is determined by using GAP version 8 from the GCG package with

20 standard penalties for DNA: GAP weight 5.00, length weight 0.300, Matrix described in Gribskov and Burgess, Nucl. Acids Res. 14(16); 6745-6763 (1986), and the degree of homology between two amino acid sequences is determined by using GAP version 8 from the GCG package (Genetics Computer Group, 575 Science Drive, Madison, Wis. 53711, USA) with standard penalties for proteins: GAP weight 3.00, length weight 0.100, Matrix

25 described in Gribskov and Burgess, Nucl. Acids Res. 14(16); 6745-6763 (1986).

A preferred minimum percentage of sequence homology is at least 70%, such as at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and at least

30 99.5%.

The invention also relates to the use of a polypeptide or nucleic acid of the invention for use as therapeutic vaccines as have been described in the literature exemplified by Lowry, D.B. et al 1999, Nature 400: 269-71.

35

A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide of the invention in an immuno assay, or a specific binding fragment of said antibody, is also a part of the invention. The antibodies can be produced by methods known to a person

skilled in the art. The polyclonal antibodies can be raised in a mammal, for example, by one or more injections of a polypeptide according to the present invention and, if desired, an adjuvant. The monoclonal antibodies according to the present invention may, for example, be produced by the hybridoma method first described by Kohler and

5   Milstein, Nature, 256:495 (1975), or may be produced by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The monoclonal antibodies may also be isolated from phage libraries generated using the techniques described by McCafferty *et al*, Nature, 348:552-554 (1990), for example. Methods for producing antibodies are described in the literature, e.g. in US 6,136,958.

10

In diagnostics, treatment or testing, an antibody, a nucleic acid fragment and/or a polypeptide of the invention can be used either alone, or as a constituent in a composition. Such compositions are known in the art, and comprise compositions in which the antibody, the nucleic acid fragment or the polypeptide of the invention is

15   coupled, preferably covalently, to at least one other molecule, e.g. a label (e.g. radioactive or fluorescent) or a carrier molecule.

The present invention is further directed to methods for using the compounds described above to therapeutically and/or prophylactically treat a patient for a diabetes related dis-

20   ease.

The methods of the present invention include the steps of: a) incorporating one or more of the compounds of the present invention in a suitable pharmaceutical carrier; and b) administering either a therapeutically effective dosage or a prophylactically effective

25   dosage of the compound or compounds incorporated in the carrier to a patient.

The term "suitable pharmaceutical carrier" refers to any carrier known in the pharmaceutical arts for administration of compounds to a patient. Any suitable pharmaceutical carrier can be used according to the present invention, so long as

30   compatibility problems do not arise.

Administration of an effective dosage to a patient can be accomplished by parenteral in-jection, such as intravenously, intrathecally, intramuscularly or intra-arterially. The com-pounds can also be administered orally or transdermally, or by any other means known

35   to those skilled in the art, e.g. by means of an inhalator or a nasal spray. Oral administration is presently preferred.

As used herein, the term "therapeutically effective amount" refers to that amount of one or more of the compounds of the present invention required to therapeutically treating a patient. Such treatment is appropriate for subjects having a diagnosed diabetes related disease. Similarly, the term "prophylactically effective amount" refers to that amount of

5   one or more of the compounds of the present invention needed to prophylactically treat a patient. Such treatment is appropriate for subjects who, for example, have not yet established any clinical symptoms of a diabetes related disease. It could be advantageous to start a prophylactic treatment as soon it is determined that the subject is in risk for developing a diabetes related disease, e.g. by means of a determination of a

10 predisposition for diabetes by having an altered level of markers.

As will be appreciated by a person skilled in the art, the dosage of compound given, the route of administration and the duration of therapy will be dependent not only the type of compound and its effectiveness in treating the disease but also upon the individual

15 being treated, taking into consideration such factors as the body weight of the patient, other therapies being employed to treat the patient, and the condition, clinical response and tolerance of the patient. Dosage, administration, and duration of therapy can be determined by one skilled in the art upon evaluation of these and other relevant factors.

EXAMPLES

20 Example 1

The aim was to describe PIPP induced by 24 hours of incubation with 150 pg/ml recombinant human IL-1ß (specific activity was 400 U/ng, Novo Nordisk, Bagsvaerd, Denmark) in neonatal BB-DP rat islets identified by 2-DGE of labelled islets (n=3 for each

25 group) [Christensen, 2000] [13]. Protein spots that significantly changed expression levels after exposure to IL-1ß were cut out of preparative gels and subjected to MS.

Preparatory 2D-gels were produced from a pool of approximately 200.000 neonatal WF islets isolated, prepared and separated on gels as described above. For localization of the

30 spots, radioactively labelled tracer islets were mixed with the non-labelled islets. Neonatal WF rat islets were used for preparatory gels because of the price and number of islets isolated per animal. The pattern seen on 2D-gels of respectively BB-DP and WF rat islets are similar. The cost of one WF rat is approximately one tenth of the price of a BB-DP rat (approximately 100 US$ each). From each rat is it possible to isolate in the range

35 of 150-200 islets.

Reagents
RPMI 1640, Hanks' balanced salt solution (HBSS) and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland). RPMI 1640 contained 11 mmol D-glucose

and was supplemented with 20 mM HEPES buffer, 100,000 IU/l penicillin and 100 mg/l
streptomycin. Other reagents: 2-mercaptoethanol, foetal calf serum (BSA), normal
human serum (NHS), Tris HCl, Tris base, glycine, (Sigma, St. Louis, USA); trichloracetic
acid (TCA), phosphoric acid, NaOH, glycerol, n-butanol, bromophenol blue, H3PO4 and

5   NaNO2 (Merck, Darmstadt, Germany); filters (HAWP 0.25 mm pore size) (Millipore,
Boston, USA); RNA'se A, DNA'se I (Worthington, Freehold, NJ, USA); [35S]-methionine
(SJ 204, specific activity: >1.000 Ci/mmol, containing 0.1% 2-mercaptoethanol), Ampli-
fy® (Amersham International, Amersham, UK); urea (ultra pure) (Schwarz/Mann,
Cambridge, MA, USA); acrylamide, bisacrylamide, 4N-tetra-methyl-ethylene-diamine

10   (TEMED), ammonium persulphate (BioRad, Richmond, CA, USA); ampholytes: pH 5-7,
pH 3.5-10, pH 7-9, pH 8-9.5 (Amasham Biotech, Sweden); Nonidet P-40 (BDH, Poole,
UK); ampholytes: pH 5-7 and sodium dodecyl sulphate (Serva, Heidelberg, Germany);
agarose (Litex, Copenhagen, Denmark); ethanol (absolute 96%) (Danish Distillers,
Aalborg, Denmark); methanol (Prolabo, Brione Le Blanc, France); acetic acid (technical

15   quality, 99% glacial) (Bie & Berntsen, Århus, Denmark) and X-ray film (Curix RP-2)
(AGFA).

Animals

Pregnant inbred WF rats were purchased from M&B, Ll. Skensved, Denmark. Four to five

20   day-old BB/Wor/Mol-BB2 (BB-DP) rats were also purcased from M&B. The rats were
picked up at M&B in the morning on the day of islet isolation, and transported in animal
transport boxes. At M&B the BB-DP rats were housed separately in a specific pathogen-
free environment.

25   *Isolation, culture and labelling of islets for preparative gels*

Islets were isolated by collagenase digestion of the pancreata from 4-5 day old WF rats
[Brunstedt, 1984][15]. After 4 days of preculture in RPMI 1640 + 10% fetal calf serum,
islets were incubated for 24 h in 37°C humidified atmospheric air in 300 µl or 3000 µl
RPMI 1640 + 0.5% normal human serum. Next islets were washed twice in HBSS and

30   labelled for 4 h at 37OC in 200 µl or 2000 µl home made methionine-free Dulbecco's
modified Eagle's medium (DMEM) with 10% dialysed NHS, and 200 µCi [35S]-methio-
nine. To eliminate 2-mercaptoethanol, [35S]-methionine was freeze-dried for at least 4 h
before labelling. After labelling, the islets were washed thrice in HBSS, the supernatant
was removed and islets were immediately frozen at -80°C. Unlabelled islets for

35   preparative gels were washed twice in HBSS and snapfrozen. For localization of the
spots, radioactively labelled tracer islets were mixed with the non-labelled islets.

Sample preparation

The frozen islets were re-suspended in 100 µl DNAseI/RNAse A solution and lysed by freeze-thawing twice. After the second thawing, the samples were left on ice for 30 min. for the digestion of nucleic acids and then freeze dried overnight. The samples were dissolved by shaking in 120 µl lysis buffer (8.5 M urea, 2% Nonidet P-40, 5%

5   2-mercaptoethanol and 2% ampholytes, pH range 7-9) for a minimum of 4 hours.

Determination of [35S]-methionine incorporation

The amount of [35S]-methionine incorporation was quantified by adding 10 µl FCS (0.2 µg/ml H2O) as a protein-carrier to 5 µl of a 1:10 dilution of each sample in duplicate,

10   followed by 0.5 ml of 10% TCA. This was left to precipitate for 30 min at 4°C before being filtered through 0.25 µm hydroxy appatit-WP (HAWP) filters. The filters were dried and placed into scintillation liquid for counting.

2-DGE and preparative gels

15   Preparative two dimensional gels (2-DG) were produced from a pool of approximately 200.000 neonatal WF rat islets isolated, cultured, labelled and separated on gels as described above. For localization of the spots, radioactively labelled tracer islets were mixed with the non-labelled islets.

The procedure has been described earlier [O'Farrell, 1977 [14]; Fey, 1984 [18]; Fey,

20   1997 [17]. Briefly, first dimension gels contained 4% acrylamide, 0.25% bisacrylamide, ampholytes and nonidet P-40. Equal amount of protein (175-200 µg for preparative gels) and counts per minute (106 cpm.) of each sample were applied to the gels. Both isoelectric focusing (IEF; pH 3.5-7) and non-equilibrium pH-gradient electrophoresis (NEPHGE; pH 6.5-10.5) gels were made. Second dimension gels contained 12,5%

25   acrylamide and 0.063% bisacrylamide and were run overnight. After electrophoresis, the gels were fixed and treated for fluorography with Amplify® before being dried. The gels were placed in contact with X-ray films and exposed at -70°C for 3 to 40 days. Each gel was exposed for at least 3 time periods to compensate for the lack of dynamic range of X-ray films.

30   Neonatal WF rat islets were used for preparative gels because of the price and higher number of islets isolated per animal. The same protein spot pattern is seen on 2-DG of respectively BB-DP and WF rat islets and the BB-DP rat originates from the WF rat. The cost of one WF rat is approximately one tenth of the price of a BB-DP rat (approximately 100 US$ each). From each rat was it possible to isolate approximately 150-200 islets.

35

Determination of Mr and pI

Mr and pI for individual proteins on the gels were interpolated from landmark proteins. Landmark proteins were determined by use of internal standards and pI calibration kits

[Fey, 1984][18]. Theoretical Mr and pI were calculated using the Compute pI/Mw tool at the ExPASy Molecular Biology Server (www.expasy.ch/tools/pi_tool.html).

Computer analysis of fluorographs

5   Computer analysis was performed using the BioImage® program 2D-Analyzer (version 6.1) [Christensen, 2000][13]. Briefly, computer analysis was performed using the BioImage® program 2D-Analyzer (version 6.1) on a Sun Ultra 1 computer. First, the fluorographs were scanned and spots identified and quantified by the 2D-Analyzer. Next, anchor points were placed on the gel (same spot in each gel was assigned the same

10   anchor-point), and an initial computer based match of the gels was performed. After computer matching, manual editing was performed to ensure correct matching of computer found spots as well as matching and quantification of spots not found by the initially computer matching. Approximately 30% of the spots were matched correct by the computer. Finally, data were extracted for calculations in the Quatro Pro®

15   spreadsheet (Borland version 4.0). To avoid the presence of duplicate spots in the IEF and NEPHGE subgroups, overlapping spots in either the basic part of IEF gels or in the acidic part of NEPHGE gels were omitted from analysis.

Protein identification by matrix-assisted laser desorption/ionization (MALDI) MS

20   Briefly, the 82 protein spots of interest were obtained by cutting them out of the dried gel using a scalpel. The proteins were enzymatically digested in the gel piece as described [Rosenfeld, 1992 [19]; Shevchenko, 1996 [20]] with minor modifications [Nawrocki, 1998][21]. The excised gel pieces were washed in 50 mM NH4HCO3/acetonitrile (60/40) and dried by vacuum centrifugation. Modified porcine

25   trypsin (12 ng/µL, Promega, sequencing grade) in digestion buffer (50 mM NH4HCO3) was added to the dry gel pieces and incubated on ice for 1 h for reswelling. After removing the supernatant, 20-40 µL digestion buffer was added and the digestion was continued at 37o C for 4-18 hours. The peptides were extracted as described [Shevchenko, 1996][20] and dried in a vacuum centrifuge. The residue was dissolved in

30   5 % formic acid and analysed by MALDI MS. Delayed extraction MALDI mass spectra of the peptide mixtures were acquired using a Bruker Reflex time-of-flight mass spectrometer (Bruker AG, Germany). Samples were prepared using α-cyano-4-hydroxy cinnamic acid as matrix [Kussmann, 1997][22]. Protein identification was performed by in silico comparison of the theoretical peptide-mass maps in the comprehensive, non-

35   redundant protein sequence database (NRDB, European Bioinformatics Institute, Hinxton, UK http://www.ebi.ac.uk) using the PeptideSearch software ([Mann, 1993][23] further developed at EMBL (Heidelberg, Germany)), SWISS-PROT (http://www.expasy.ch), PIR (http://www.sanger.ac.uk/DataSearch), NIH and GENEBANK (http://www.ncbi.nlm.nih.gov). The protein identifications were examined

using the "second pass search" feature of the software and critical evaluation of the peptide mass map as described [Jensen, 1998][24].

Protein information

5   Information about the identified proteins known and putative biological functions were found at The ExPASy Molecular Biology Server (http://www.expasy.ch) and at The National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

Statistical analysis

10   Student's t-test was applied and p < 0.01 was chosen as level of significance.

Results

All 82 significantly changed protein spots were re-identified in preparative gels of neonatal WF islets and could be excised from the gels for MS identification. Positive

15   identification was obtained for a total of 45 different proteins from 51 of the 82 spots (table 1). Six spots contained 2 identifyable proteins (NEPHGE match no. 339, 370 and IEF 955, 248, 1136, 660). Some proteins were present in more than one spot: Three proteins were present in 2 spots (Heterogeneous nuclear ribonucleoproteins A2/B1, calreticulin and NADH dehydrogenase), 3 proteins were present in 3 spots (malate

20   dehydrogenase precursor (mitochondrial), IgE binding protein and glucose-regulated protein 78 (GRP 78)) and one protein, tubulin beta-5 chain was present in 4 spots. The presence of the same protein in more than one spot suggests that the protein exists in different posttranslatory modified or degraded forms. Positive identification was not obtained for 31 protein spots either due to spectra with no data base match (n=16) or

25   low abundance of peptides in the excised and digested spots (n= 15) (table 2). Thus, success rate of positively identified protein spots was 62 % (51 spots out 82). A similar result, 60 spots identified out of 105 (58 %) were obtained from WF rat islets exposed to IL-1ß, corresponding to 57 different proteins [Mose Larsen, 2001][7]. Thirteen proteins identified in BB-DP and WF rat islets were identical (NEPHGE match no. 223, 231, 370,

30   146, 381 (2 spots), IEF match no. 1127, 955, 552, 138 (3 spots), 1136, 8580, 68, 62) (table 1).

In table 1, the protein Mr's and pI's observed on the gels are presented together with the computed theoretical Mr and pI values. Differences in observed and computed values of

35   Mr and pI may be due to posttranslatory modifications, degradation or pro- or pre-pro forms of proteins since the theoretical values are based on the open reading fame only. For example calreticulin is present in 2 spots with a Mr of respectively 39.9 kDa and 120.7 kDa whereas the theoretical value is 48 kDa. Nevertheless for most proteins there

are only minor differences between observed Mr/pI values and theoretical values (table 1).

In total, 45 different proteins and 12 modified forms of some of these proteins have been identified to change expression level after exposure to IL-1β. Based upon known or

5   putative functions the 45 identified proteins (with modified forms 57) have been grouped as follows (in brackets, number of different proteins): proteins involved in a) energy transduction and redox potentials (n=8), b) glycolytic and Krebs cycle enzymes (n=6), c) protein synthesis (including purin/pyrimidine synthesis, DNA/RNA synthesis, RNA processing and amino acid metabolism), chaperoning and protein folding (n=14), d)

10   signal transduction, regulation, differentiation and apoptosis (n=6), e) cellular defence (n=2) and f) other functions (n=9) (table 1).

LEGENDS

Table 1

Identified protein spots in neonatal BB-DP rats islets after exposure to IL-1β. Changes in

15   protein expression level are expressed as % IOD in neonatal BB-DP islets after IL-1β incubation *in vitro* compared to BB-DP islets without IL-1β. Match numbers are arbitrary numbers given by the computer and corresponds to the number of the spot in the gel. % IOD refers to the integrated optic density on the control gels. % IOD ratio refers to the ratio of integrated optical density between gels compared. Numbers below 1 indicate

20   proteins down-regulated and numbers above 1 are up-regulated in islets exposed to IL-1β. The proteins spots are ordered according to functional groups and % IOD ratio. First spots on the NEPHGE (prefix N) side and next the IEF (prefix I) side. Protein name refers to the name found in the NCBI database (http://www.ncbi.nlm.nih.gov) through the database accession number. Given is the Mr and pI obtained directly by the gel analysis

25   and the calculated theoretical Mr and pI calculated from the amino acid sequence. In some spots more than one protein was identified. These proteins are marked with *. Proteins mentioned more than once are found in more than one spot. Proteins previously identified as changes in expression in WF rat islets exposed to IL-1β as well [Mose Larsen, 2001][7] are marked with #. Proteins that have not previously been described

30   or supposed to be present in islets are marked with $.

Table 2

In table 2, unidentified protein spots with significant change in expression level expressed as % IOD in neonatal BB-DP islets after IL-1β incubation *in vitro* are compared

35   to BB-DP islets without IL-1β. Match numbers are arbitrary numbers given by the computer and corresponds to the number of the spot in the gel. % IOD ratio refers to the ratio of integrated optical density between gels compared. Numbers below 1 are down-regulated and numbers above 1 are up-regulated in islets exposed to IL-1β. Protein spots

are ordered numerically. First spots on the IEF (prefix I) side and next on the NEPHGE
(prefix N) side. The Mr in kDa and pI obtained directly from the gels are given. Where
spectra were obtained by tryptic digestion, their Mr of the peptides is given in table 3 and
their spectra given in pairs of figures as shown.

5

Figure 1

Fluorograph of a two-dimensional gel of neonatal BB-DP rat islets of Langerhans
incubated for 24 hours in control medium followed by 4 hours labeling with [35S]-
methionine. The gel shown is representative of 3 experiments. The marked proteins

10   represents proteins changing level of expression during incubation with IL-1B. IEF gel
(pH 3.5-7) on the right side and NEPHGE gel (pH6.5-10.5) on the left side. The numbers
correspond to the proteins in table 1 and 2.

Figures 5-21

15   Spectra of the proteins in table 3. Each spectrum is presented firstly as a total spectrum
(e.g. figure 5A) and thereafter as enlarged regions so it is possible to read all the
numbers and see the peak that they refer to (e.g. figure 5B, figure 5C, etc.). Thus, the
spectra for the first protein in table 3 are called figure 5A, figure 5B, figure 5C and figure
5D.

Table 1

Identification of proteins induced by IL-1β in BB-DP rat islets

Gel match No	% IOD	% IOD ratio	Protein name	Function	Database acc. no.	Mr.	Theoretical Mr.	pI	Theoretical pI
Energy transduction and redox potentials									
N 318	0.165	0.56	Alcohol dehydrogenase	Redox potential	gi 1703237	42.4	36.4	7.8	6.8
N 231 #	0.041	0.51	ATP synthase alpha chain, mitochondrial precursor	Energy transduction	gi 114523	51.1	58.8	7.9	9.2
N 370 * #	2.055	0.24	L-3-hydroxyacyl-CoA dehydrogenase precursor	Energy transduction, redox potential	gi 5353512	36.5	34.3	8.4	8.9
N 1247	0.084	0.20	Carbonyl reductase	Redox potential	gi 1352258	37.7	30.4	8.2	8.2
I 955 * #	0.104	2.41	Creatine kinase-B	Energy transduction	gi 203476	49.3	42.7	5.4	5.3
I 1136 *	0.405	0.45	H+ transporting ATP synthase	Energy transduction	gi 92350	52.9	56.4	4.8	5.2
I 706	0.086	0.43	NADH dehydrogenase	Energy transduction	gi 4826856	86.7	79.6	5.3	5.8
I 705	0.084	0.35	NADH dehydrogenase	Energy transduction	gi 4826856	87.3	79.6	5.2	5.8
I 552 #	0.132	0.07	ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta polypeptide, 56/58	Energy transduction, intracellular environment	gi 4502309	57.6	57.0	5.3	5.5

			kD, isoform 1						
Glycolytic and Krebs Cycle enzymes									
I 629	0.065	2.15	Glucose-6-phosphate dehydrogenase	Energy generation	gi 204197	61.8	59.2	6.0	6.0
I 1127 #	0.067	0.29	Glyceraldehyde-3-phosphate dehydrogenase	Energy generation	gi 203142	54.4	35.7	6.7	8.4
N 223 #	0.379	0.52	Pyruvate kinase M2 isozyme	Energy generation	gi 1346398	55.6	57.6	8.0	7.4
N 88	0.288	0.41	Aconitate hydratase, mitochondriel precursor	Energy generation	gi 1351857	77.8	85.4	7.3	8.1
N 272	0.433	0.48	Isocitrate dehydrogenase 2, mitochondrial	Energy generation	gi 6680343	45.9	58.7	8.4	8.9
N 1325	0.137	0.38	Malate dehydrogenase precursor, mitochondrial	Energy generation	gi 319830	41.9	35.5	9.0	8.9
N 336	0.118	0.36	Malate dehydrogenase precursor, mitochondrial	Energy generation	gi 319830	42.7	35.5	9.0	8.9
N 339 *	0.830	0.28	Malate dehydrogenase precursor, mitochondrial	Energy generation	gi 319830	40.4	35.5	8.7	8.9
Protein synthesis (incl. DNA/RNA processing and synthesis, purin/pyrimidine synthesis, amino acid metabolism) chaperones and protein folding									
N 146 #	0.590	0.44	5-aminoimidazole-4-	Purine/pyrimidine synthesis,	gi 2541906	64.8	64.2	6.9	6.7

ID			Protein	Function	gi				
			carboxamide ribonucleotide formyltransferase	DNA/RNA synthesis					
I 248 *	0.030	0.48	Adenosine phosphoribosyltransferase	Purine/pyrimidine synthesis, DNA/RNA synthesis	gi 543829	21.5	19.5	5.7	6.2
I 248 *	0.030	0.48	UMP-CMP kinase	Purine/pyrimidine synthesis, DNA/RNA synthesis	gi 5730476	21.5	22.2	5.7	5.4
N 1414	0.070	0.41	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA-processing	gi 6647752	42.1	36.0	8.0	8.7
N 339*	0.830	0.28	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA-processing	gi 6647752	40.4	36.0	8.7	8.7
N 1401	0.482	0.17	M4 protein deletion mutant	RNA-processing	gi 3126878	64.4	77.5	8.5	8.9
N 317	0.071	0.35	Aspartate transaminase, cytosolic	Amino acid metabolism	gi 91997	44.9	46.2	7.9	6.3
N 1322	0.054	0.15	Aspartate aminotransferase, mitochondrial precursor	Amino acid metabolism	gi 112987	47.6	47.3	9.1	9.1
I 237	0.534	0.12	60 ribosomal protein L11	Ribosome protein	gi 971761	20.9	18.8	4.4	9.9
I 653	0.722	1.52	Calreticulin	Chaperone, transcription factor	gi 6680836	120.7	48.0	3.7	4.3
I 585	0.061	2.82	Calreticulin	Chaperone, transcription factor	gi 6680836	39.9	48.0	6.0	4.3
I 138 #	0.881	1.79	78 kD glucose-regulated protein precursor (GRP 78) (HSP70 family)	Chaperone, signal transduction	gi 121574	78.4	72.3	4.8	5.1

33

ID			Protein	Function	gi				
I 660 ##	0.208	0.18	78 kD glucose-regulated protein precursor (GRP 78) (HSP70 family)	Chaperone, signal transduction	gl 121574	70.2	72.3	4.8	5.1
I 6347 #	0.173	0.39	78 kD glucose-regulated protein precursor (GRP 78) (HSP70 family)	Chaperone, signal transduction	gl 121574	65.1	72.3	4.9	5.1
I 1136 ##	0.405	0.45	Probable protein disulfide Isomerase P5 precursor	Chaperone, signal transduction	gl 2501206	52.9	47.2	4.8	5.0
I 8580 #	0.034	0.38	Protein disulfide Isomerase	Chaperone, signal transduction	gl 91897	60.2	56.6	6.0	5.9
I 68 #	0.084	0.23	Endoplasmic reticulum protein ERP 29 precursor	Chaperone, signal transduction	gl 2507015	25.9	28.6	6.3	6.2
I 660 *	0.208	0.18	Endoplasmin precursor (HSP90 family)	Chaperone, signal transduction	gl 119362	70.2	92.5	4.8	4.7

Signal transduction, regulation, differentiation and apoptosis

ID			Protein	Function	gi				
N 381 #	0.495	3.93	Galectin-3 (IgE binding protein)	Differentiation, apoptosis	gl 204728	36.8	27.1	7.9	8.2
N 377 #	3.926	2.11	Galectin-3 (IgE binding protein)	Differentiation, apoptosis	gl 204728	36.9	27.1	8.3	8.2
N 398 #	0.007	28.15	Galectin-3 (IgE binding protein)	Differentiation, apoptosis	gl 204728	31.4	27.1	8.8	8.2
N 370 *	2.055	0.24	Voltage dependent anion channel	Cellular transport, apoptosis	gl 4105605	36.5	32.4	8.4	8.3
I 62 #	0.060	3.24	TOAD 64 (Dihydropyrimidinase related protein-2)	Differentiation, signal transduction	gl 1351260	69.5	62.3	6.3	6.0
I 1153	0.094	1.42	Eukaryotic Initiation factor 4A-	Signal transduction	gl 729821	44.8	46.8	6.3	6.1

34

ID			Name	Function	gi number				
I 217 $	0.005	23.63	Craniofacial development protein 1 like NUK	Eukaryotic organogenesis	gl 5453567	19.9	33.6	5.2	4.8
I 293	0.686	0.24	Secretagogin	Proliferation, differentiation, signal transduction	gl 3757661	25.8	32.1	5.5	5.3
Cellular defence									
N 180	0.096	3.03	Catalase	Cellular defence	gl 115707	61.9	59.6	7.5	7.2
I 2420	0.026	0.13	Glutathione synthetase	Cellular defence	gl 1170038	51.9	52.3	5.3	5.5
Other functions									
I 1096	0.169	0.17	Neuroendocrine convertase 1	Hormone processing	gl 128001	73.8	84.1	4.7	5.8
I 6363	0.229	0.12	Neuroendocrine convertase 2	Hormone processing	gl 128004	72.1	70.8	5.2	5.9
N 279	0.271	0.33	Beta-alanine oxoglutarate aminotransferase	GABA catabolism	gl 3046865	49.8	56.5	8.0	8.5
I 1202 $	0.035	0.03	25-Dx	Progesterone receptor	gl 1518818	24.3	21.5	4.2	4.5
N 432	0.086	0.17	Amyloid beta-peptide binding protein	Hormone metabolism, apoptosis	gl 2961553	30.9	27.1	8.5	8.9
I 299	0.129	0.24	5-aminolevulinate synthase precursor	Heme synthesis	gl 599830	26.8	71.1	4.8	9.0
I 955 *	0.104	2.41	Cytokeratine 8 polypeptide	Cellular structure	gl 203734	49.3	53.9	5.4	5.8
I 2410	0.331	0.46	Tubulin beta-5 chain	Cellular structure	gl 135471	58.1	49.7	4.6	4.8

36

I 6379	0.383	0.44	Tubulin beta-5 chain	Cellular structure	gi 135471	65.7	49.7	4.5	4.8
I 590	0.485	0.41	Tubulin beta-5 chain	Cellular structure	gi 135471	64.9	49.7	4.5	4.8
I 1139	0.561	0.37	Tubulin beta-5 chain	Cellular structure	gi 135471	59.5	49.7	4.6	4.8
N 1512 $	0.038	0.14	Immediate-early protein 1 rat cytomegalovirus	Virus protein, transcription factor	gi 543613	51.6	66.7	8.4	4.7

Table 2

Gel match No	%IOD ratio	Protein identification	Apparent Mr.	Apparent pI	Peptide fragment list	Spectrum shown in figures
I 75	0.43	Weak spectrum no id	62.2	6.5	Table 3	5A-5D
I 242	0.08	No spectrum	20.6	5.0		
I 266	1.77	Weak spectrum no id	24.8	4.8	Table 3	6A-6D
I 270	0.22	Good spectrum no id	22.5	5.4	Table 3	7A-7C
I 275	0.26	No spectrum	22.7	6.0		
I 292	0.08	Weak spectrum no id	24.8	4.5	Table 3	8A-8E
I 327	0.43	No spectrum	24.4	6.5		
I 408	0.70	Weak spectrum no id	32.5	5.9	Table 3	9A-9D
I 418	0.18	Good spectrum Protein Name: Pyridoxal kinase	33.2	6.3	Table 3	10A-10E
I 545	0.11	No spectrum	54.8	4.5		
I 683	3.36	Weak spectrum no id	72.9	6.3	Table 3	11A-11E
I 712	0.26	No spectrum	90.1	5.4		
I 838	0.20	No spectrum	65.1	4.9		
I 961	2.58	Good spectrum no id	52.0	5.1	Table 3	12A-12E
I 1196	0.22	No spectrum	24.4	6.1		
I 6585	0.42	No spectrum	30.2	6.0		
I 7495	0.04	No spectrum	30.2	5.6		
I 8264	21.03	Good spectrum no id	13.7	6.6	Table 3	13A-13H
I 8311	0.08	Weak spectrum no id	21.3	4.5	Table 3	14A-14C
I 8330	0.27	No spectrum	25.5	4.4		
N 68	0.12	Weak spectrum no id	89.1	6.4	Table 3	15A-15C
N 207	0.38	No spectrum	57.4	8.8		
N 212	0.33	Weak spectrum no id	57.0	8.5	Table 3	16A-16E
N 268	3.13	Good spectrum no id	47.7	8.6	Table 3	17A-17E
N 281	9.29	No spectrum	46.1	8.0		
N 284	5.38	No spectrum	46.9	7.7		
N 403	0.31	Weak spectrum no id	31.8	8.5	Table 3	18A-18C
N 435	6.03	Weak spectrum no id	29.0	8.3	Table 3	19A-19C
N 509	1.50	Weak spectrum no id	23.9	7.9	Table 3	20A-20C
N 1282	28.67	No spectrum	31.8	8.8		
N 1355	0.25	Good spectrum no id	44.1	8.0	Table 3	21A-21D

Table 3. Molecular weights of the tryptic peptide fragments of novel proteins for which spectra were obtained but for which no match existed in the current databases.

IEF proteins

Protein I 75

670.5570	684.5477	686.5041	728.5521	873.5858	889.6078
908.6622	922.6956	944.6519	950.7223	966.7041	984.6715
1004.5717	1077.7616	1086.6330	1132.6399	1166.6526	1168.6858
1185.7015	1193.6042	1242.5950	1263.6976	1272.7057	1342.0115
1355.8121	1357.8145	1442.7404	1455.7503	1488.8207	1524.8321
1564.7202	1607.8029	1615.8367	1716.8597	1861.9241	1940.9596
2034.8915	2053.0293				

Protein I 266

728.5354	831.5264	873.5511	886.4743	908.6607	918.4865
922.6664	944.6254	949.5555	980.5395	1057.5931	1084.5941
1193.6314	1234.6795	1245.5738	1261.5759	1358.7697	1404.6458
1434.7914	1512.7986	1561.9757	1601.7975	1617.8125	1650.9545
1716.8823	1783.8355	1815.9457	1837.9834	1851.9422	1887.9667
1917.9224	1933.9909	1997.9097	2046.0106	3606.3085	

Protein I 270

679.5086	728.5476	854.4555	908.6556	944.5150	950.7108
988.5306	1018.5908	1021.5312	1081.5950	1088.4804	1154.6793
1457.7506	1491.7937	1502.7706	1560.8159	1563.7089	1675.8092

Protein I 292

881.3373	1168.6586	1189.6091	1205.6194	1263.6377	1419.7204
1487.7331	1545.7266	1547.7188	1576.8979	1625.8683	1638.8148
1687.8336	1819.8460	1851.8649	1940.8958	1977.9077	1987.9647
2082.9463	2536.1013				

Protein I 408

679.4761	728.5525	796.4827	1037.5187	1062.5377	1090.5288
1126.4991	1172.5254	1193.5959	1234.6704	1256.6646	1263.6611
1349.7196	1371.7023	1393.7119	1397.7314	1434.7747	1458.7111
1636.9152	1638.8883	1657.7814	1838.9477	1851.8797	1940.9565
2034.0037	2367.2130	2399.0118			

Protein I 418

964.5183	1037.5627	1064.5737	1137.6832	1172.5764	1192.6737
1235.6392	1250.6431	1263.6860	1269.7053	1297.6467	1343.8145
1351.6546	1434.8046	1682.0241	1766.8335	1780.9714	1794.9118
1810.1236	1833.9644	1881.9621	2010.0111	2490.2406	

Protein I 683

679.4642	686.4822	728.5341	740.4581	754.4010	831.5268
908.6695	922.6996	924.6633	927.5649	942.6207	944.6316
950.7218	964.7080	966.7058	989.5694	1027.7101	1057.5632
1077.7748	1126.5526	1129.7359	1131.7469	1192.6919	1209.6791
1235.5576	1267.7130	1299.9801	1335.9426	1427.7586	1454.7041
1459.7211	1545.7712	1560.8588	1562.8589	1586.8190	1648.8764
1670.9380	1724.8275	1940.9525	1948.9614	1975.9984	

Protein I 961

759.3961	890.4867	897.4147	973.4941	984.5204	1010.5798
1121.5470	1181.5950	1213.5632	1234.6504	1263.6617	1398.6162
1414.6316	1415.7328	1424.6398	1434.7767	1445.7310	1458.6971
1483.7347	1527.7932	1558.8253	1561.8434	1568.7826	1601.7848
1614.8114	1657.8223	1837.9453	1859.9258	1930.8506	1940.9234
2510.0925					

Protein I 8264

642.5045	658.5016	686.4927	728.5465	740.4921	774.4709
780.5196	831.5513	865.5687	867.5099	869.6086	873.5823
894.6489	908.7021	922.6901	924.6790	936.6994	942.6411
944.6494	950.7201	964.6993	966.7231	984.7021	1027.7337
1033.8070	1077.7870	1113.7539	1115.7545	1119.7935	1129.7573
1131.7649	1165.7695	1167.7520	1172.7909	1299.9896	1316.0267
1333.9461	1335.9603	1342.0281	1427.7685	1454.7054	1521.0357
1523.0652	1562.8953	1724.8181	2131.0723		

Protein I 8311

850.5019	856.5146	869.4636	918.4998	944.5313	969.5587
1057.5591	1067.6079	1126.5398	1194.6171	1211.6538	1225.6115
1234.6546	1259.6481	1390.7952	1547.8033	1574.8037	1940.9446

NEPHGE Proteins

Protein N 68

934.5011	973.5176	995.5134	1037.4985	1057.5457	1067.5389
1090.5301	1165.5666	1193.6021	1201.6298	1234.6758	1254.6088
1263.6946	1357.7112	1372.7550	1383.6956	1434.7743	1493.7441
1838.9202					

Protein N 212

679.4801	728.5553	754.4194	831.5235	856.5218	870.5557
908.6769	922.7039	942.6525	944.6679	950.7372	964.7121

966.7143	976.5887	984.7234	1025.6127	1029.5839	1057.5669
1077.7906	1094.5759	1125.6473	1263.6958	1266.7342	1274.7816
1277.7181	1281.6434	1299.9767	1306.6874	1314.8066	1367.8446
1425.7379	1427.7699	1437.8714	1545.7752	1659.8590	1940.9373

Protein N 268

1127.5909	1143.0754	1150.0510	1179.6245	1263.6834	1329.7091
1342.7220	1377.7676	1389.7560	1398.7576	1448.7888	1469.7860
1488.7963	1537.7106	1545.7932	1567.7595	1652.8691	1822.9858
1888.9980	1907.0101	1940.9455	1997.8974	2091.0940	2123.0874
2483.2269	2536.1216	3039.3320	3131.4566	3191.3283	

Protein N 403

832.4770	909.4422	923.4959	944.5214	976.4972	1013.4648
1036.4942	1125.4947	1158.5404	1188.5988	1232.5625	1248.5905
1263.6708	1328.6698	1350.6660	1527.7290	1545.7385	1687.9345
1695.7709					

Protein N 435

1033.5528	1036.5348	1146.5669	1219.6851	1234.6535	1263.6714
1270.7097	1350.6636	1416.7296	1434.7634	1444.7664	1469.7677
1510.6958	1545.7626	1592.7967	1645.9039	1687.9633	1859.9505
2025.9711					

Protein N 509

832.4913	908.6967	944.6603	950.7336	1111.6071	1125.5858
1127.5990	1142.5584	1143.5936	1175.6808	1204.5361	1263.7111
1337.6739	1340.7881	1378.7656	1652.8528	1688.0168	

Protein N 1355

834.5078	864.5228	880.4829	930.4477	1112.6013	1127.6663
1165.5930	1167.6745	1223.7230	1233.6671	1305.7169	1326.8050
1338.6753	1364.7585	1367.7510	1382.6926	1433.8208	1467.8768
1501.9072	1580.7527	1582.8264	1634.8417	1758.3905	1762.9243
1768.9738	1797.9604	2033.9419			

These values are given in Daltons as obtained from the mass spectrometer. Known tolerances for these values will be known to anyone skilled in the art. Peptides from trypsin, keratin and other common contaminating proteins have been removed.

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CLAIMS

1. A method for diagnosing diabetes in a mammal, such as a human, the method
comprising determining the presence, absence or level of expression of at least one
5   marker protein in a biological sample from said mammal, wherein the marker protein is
selected from the group consisting of:
a)   a protein which is defined in table 1, 2 and/or 3; and
b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as
to have at least 80% sequence homology with a protein of table 1, 2 or 3.
10

2. A method according to claim 1, wherein the method comprises establishing the
increased expression of at least one marker protein (an up-regulated marker protein)
selected from the group consisting of:
a)   a protein which is defined in table 1 and/or 2, and marked by an up arrow; and
15 b)   a protein which is a modification or a derivative of a protein in a), so as to have at
least 80% sequence homology with a protein in a).

3. A method according to any one of claims 1 or 2, wherein the biological sample is se-
lected from the group consisting of urine, blood, saliva, lymphatic fluids, and tissue.
20

4. A method according to claim 3, wherein the tissue is pancreatic tissue.

5. A method for determining the predisposition for diabetes in a mammal, such as a
human, the method comprising determining the presence, absence or relative level in a
25 biological sample originating from the mammal of at least one marker protein, wherein the
marker protein is selected from the group consisting of:
a)   a protein which is defined in table 1, 2 and 3; and
b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as
to have at least 80% sequence homology with a protein of table 1, 2 or 3.
30

6. A method for determining the predisposition in a human for diabetes, the method com-
prising:
a)   determining the increased expression of at least one marker protein in a biological
sample originating from the human, said marker protein selected from the group
35 consisting of: i) an up-regulated protein defined in table 1, 2 and/or 3, and ii) a protein
which is a modification or a derivative of a protein in i), so as to have at least 80%
sequence homology with a protein in i);
b)   determining the decreased expression in a biological sample from the human of at
least one marker protein, said marker protein being selected from the group consisting of:

i) a down-regulated protein defined in table 1, 2 and/or 3 and ii) a protein which is a modification or a derivative of a protein in i), so as to have at least 80% sequence homology with a protein in i) ; or

c)   combinations of the determinations in a) and b).

7. A method according to any one of claims 1, 5 or 6, wherein the at least one marker protein is selected from the group consisting of:

a)   one or more proteins present in a significantly lower or significantly higher amount on a polyacrylamide gel of proteins from said biological sample in relation to a control;

b)   one or more proteins present on a polyacrylamide gel of proteins from said biological sample and absent on polyacrylamide gel of proteins of a control; and

c)   one or more proteins absent on a polyacrylamide gel of proteins from said biological sample and present on polyacrylamide gel of proteins of a control.

8. A method of treating diabetes in a mammal, such as a human, comprising altering the expressing of a least one marker protein selected from the group consisting of:

a)   a protein which is defined in table 1, 2 and/or 3; and

b)   · a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3.

9. A method of treating diabetes in a mammal, such as human, comprising administering a least one compound selected from the group consisting of:

a)   a protein which is defined in table 1, 2 and/or 3;

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3;

c)   a protein having the same function as a protein in a) and/or b);

d)   a nucleotide sequence coding for a protein in a), b) or c);

e)   an antibody for a protein of a), b) or c);

f)   a nucleic acid fragment capable of binding to a protein of a) b) or c); and

g)   a compound capable of binding to a protein of a), b) or c)

to said human.

10. A method of preventing or delaying the onset or of diabetes in a mammal, e.g. a human, comprising administering:

a)   a protein which is defined in table 1, 2 and/or 3;

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3;

c)   a mimic of the protein defined in a) and b);

d)   a nucleotide sequence coding for a protein defined in a) and/or b);

e)   an antibody for a protein defined in a) and/or b);

f)   a nucleic acid fragment capable of binding to a protein of a) and/or b); and/or

g)   a compound capable of binding to a protein defined in a) and/or b)

to said human.

5

11. A method of determining the likelihood of an agent to have a therapeutic effect in the treatment of diabetes comprising determining the relative level of expression of one or more proteins from the group consisting of:

a)   a protein which is defined in table 1, 2 and/or 3; and

10 b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3

before and after exposing a test model to said agent.

12. A method of determining the effect of a compound in the treatment of diabetes com-

15 prising determining the level of expression of proteins of one or more proteins from the group consisting of:

a)   a protein which is defined in table 1, 2 and/or 3; and

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3.

20

13. A method of determining the level of effect of a compound used in the treatment of diabetes comprising determining the level of expression of one or more proteins from the group consisting of:

a)   a protein which is defined in table 1, 2 and/or 3; and

25 b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3

before and after exposing a test model to said agent.

14. A method of determining the nature or cause of diabetes in a mammal, such as a

30 human, having or susceptible to said disease comprising establishing the level of expression of a protein from the group consisting of:

a)   a protein which is defined in table 1, 2 and/or 3; and

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3

35 in relation to a model.

15. A substantially pure protein selected from the group consisting of:

a)   a protein of table 1, 2 and 3; and

b)   a protein with at least 80% sequence homology with a protein in a).

16. A nucleic acid fragment comprising a nucleotide sequence which codes for a protein defined in table 1, 2 or 3 or a protein with at least 80% sequence homology therewith.

5 17. A nucleic acid fragment which hybridizes with a nucleic acid fragment according to claim 16 or a part thereof or with its complementary strand.

18. Use of a nucleic acid fragment according to any one of claims 16-17 for detecting the presence of a peptide from the group consisting of:

10 a)   a protein which is defined in table 1, 2 and/or 3; and

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3.

19. An antibody able to bind to a protein from the group consisting of:

15 a)   a protein which is defined in table 1, 2 and/or 3; and

b) '   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3.

20. An antibody according to claim 19 which is a polyclonal antibody.

20

21. An antibody according to claim 19 which is a monoclonal antibody.

22. Use of a antibody according to any one of claims 19 to 21 for detecting the presence of a protein from the group consisting of:

25 a)   a protein which is defined in tables 1, 2 and/or 3; and

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3.

23. A test kit for diagnosing diabetes or a genetic predisposition for diabetes in a mammal,

30 such as a human, comprising:

a)   a binding means which specifically binds to at least one marker protein selected from the group consisting of: i) a protein which is defined in table 1, 2 and/or 3; and ii) a protein which is a modification or a derivative of a protein in i), so as to have at least 80% sequence homology therewith; or which binding means is an antibody for at least one said

35 marker protein, a nucleic acid fragment capable of binding to at least one said marker protein, or a compound capable of binding to at least one said marker protein;

b)   a means for detecting binding, if any, or the level of binding, of the binding means to at least one of the marker proteins or to at least one of the nucleic acid fragments, and

c)   a means for correlating whether binding, if any, or the level of binding, to said
binding means is indicative of the individual mammal having a significantly higher like-
lihood of having diabetes or a genetic predisposition for having diabetes.

5   24. A method for determining the effect of a substance, the method comprising using a
mammal, e.g. a human, which has been established to be an individual having a high
likelihood of having diabetes or a genetic predisposition for having diabetes by use of a
method according to any one of claims 1 to 7, the method comprising administering the
substance to the individual and determining the effect of the substance.

10

25. A pharmaceutical composition which comprises:
a)   a substance which is capable of regulating the expression of a nucleic acid frag-
ment coding for at least part of a protein selected from the group consisting of: i) a protein
which is defined in table 1, 2 and/or 3, and ii) a protein which is a modification or a

15 derivative of a protein in i), so as to have at least 80% sequence homology with a protein
of i);
b)   a marker protein selected from the group consisting of: i) a protein which is defined
in table 1, 2 and/or 3, and ii) a protein which is a modification or a derivative of a protein
of i), so as to have at least 80% sequence homology with a protein of i);

20 c)   a derivative, homologue or mimic of a protein selected from the group consisting
of: i) a protein which is defined in table 1, 2 and/or 3, and ii) a protein which is a
modification or a derivative of a protein in i), so as to have at least 80% sequence
homology with a protein of i);
d)   an antibody for a protein selected from the group consisting of: i) a protein which is

25 defined in table 1, 2 and/or 3, and ii) a protein which is a modification or a derivative of a
protein in i), so as to have at least 80% sequence homology with a protein of i);
e)   a nucleic acid fragment capable of binding to a marker protein selected from the
group consisting of: i) a protein which is defined in table 1, 2 and/or 3, and ii) a protein
which is a modification or a derivative of a protein in i), so as to have at least 80%

30 sequence homology with a protein of i); and/or
f)   a compound capable of binding to a marker protein selected from the group
consisting of: i) a protein which is defined in table 1, 2 and/or 3, and ii) a protein which is
a modification or a derivative of a protein in i), so as to have at least 80% sequence
homology with a protein of i).

35

26. A pharmaceutical composition according to claim 25 for use as a medicament, such as
for treatment or prophylaxis of diabetes.

27. Use of a compound selected from the group consisting of:

a)   a protein which is defined in table 1, 2 and/or 3;

b)   a protein which is a modification or a derivative of a protein of table 1, 2 or 3, so as to have at least 80% sequence homology with a protein of table 1, 2 or 3;

c)   a protein having the same function as a protein in a) and/or b);

d)   a nucleotide sequence coding for a protein in a), b) or c);

e)   an antibody for a protein of a), b) or c);

f)   a nucleic acid fragment capable of binding to a protein of a) b) or c); and

g)   a compound capable of binding to a protein of a), b) or c)

for the manufacture of a medicament for the treatment or prophylaxis of diabetes.

Figure 1 NEONATAL BB-DP rat islets
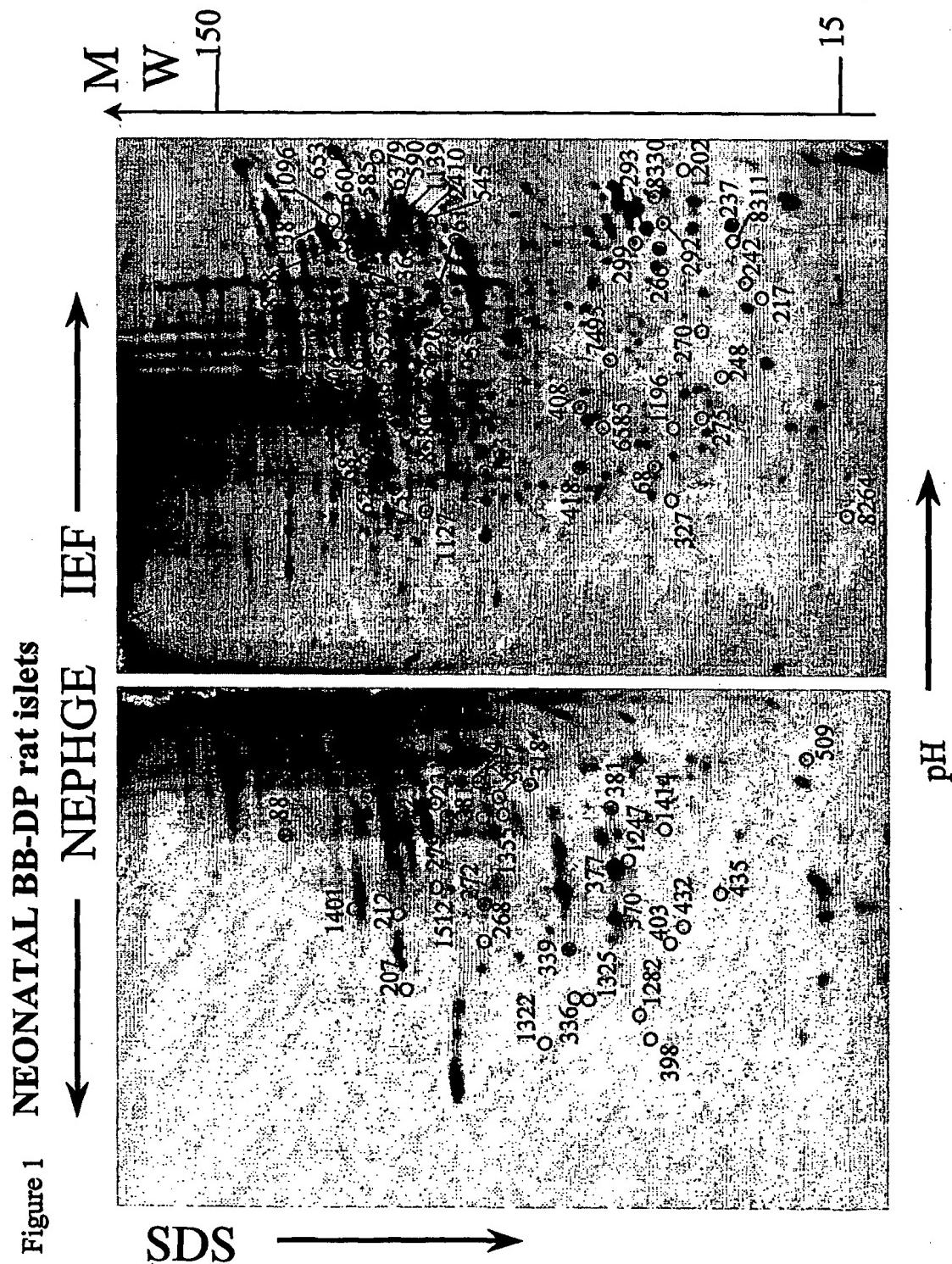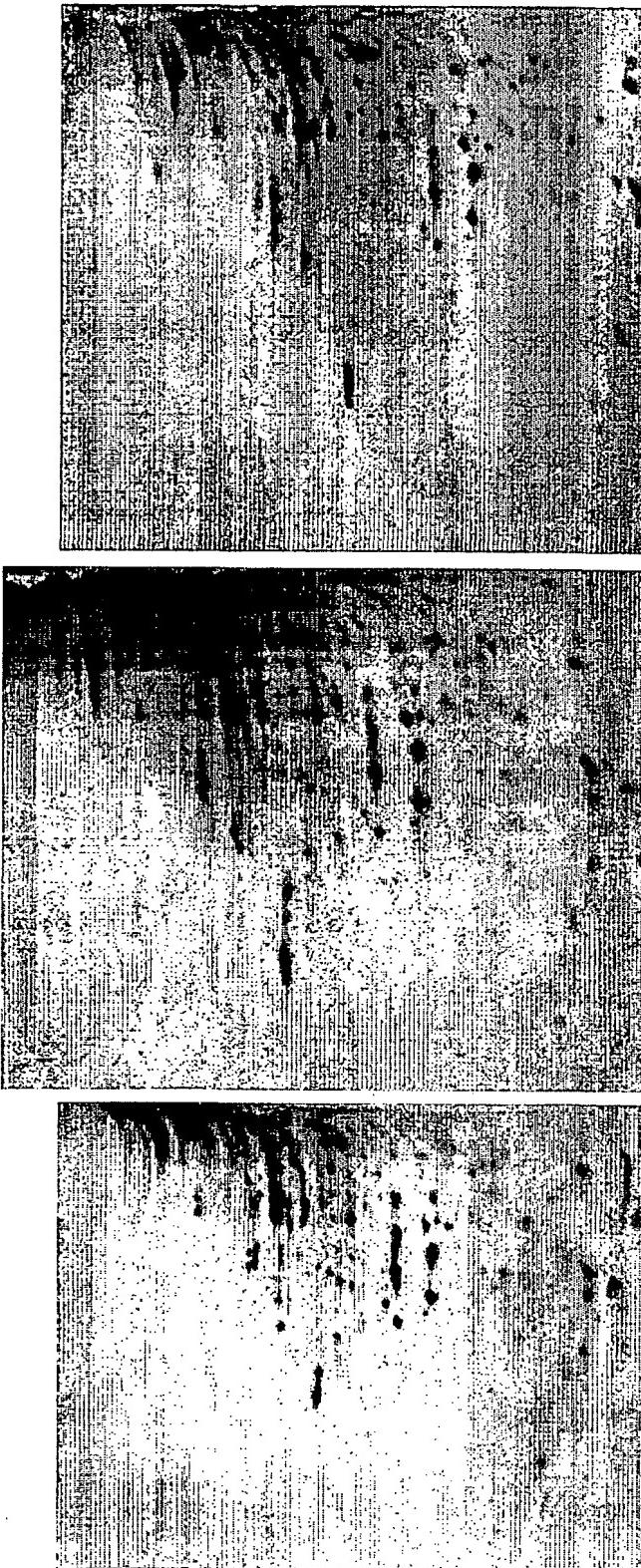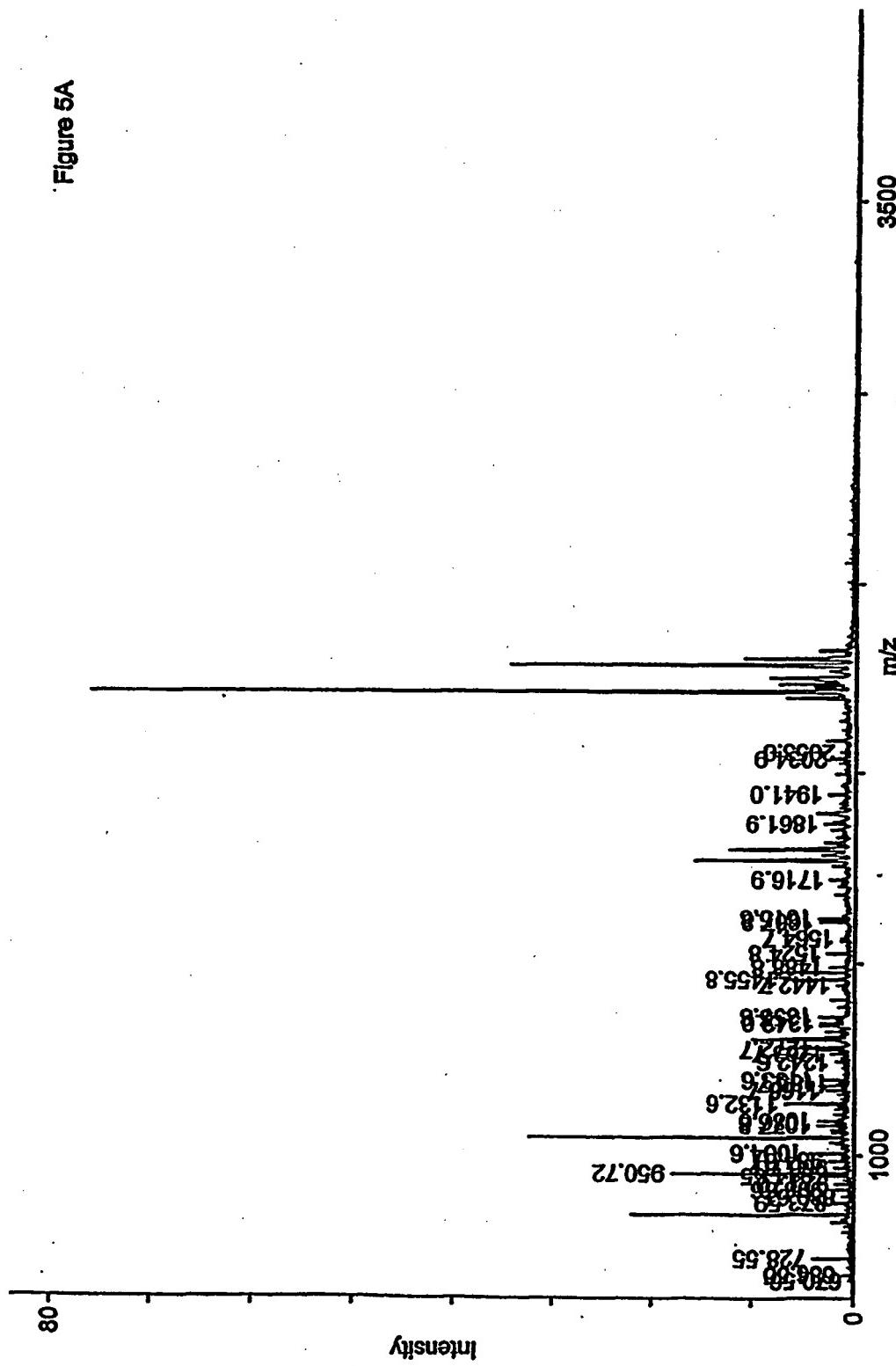
Figure 2

Figure 5A

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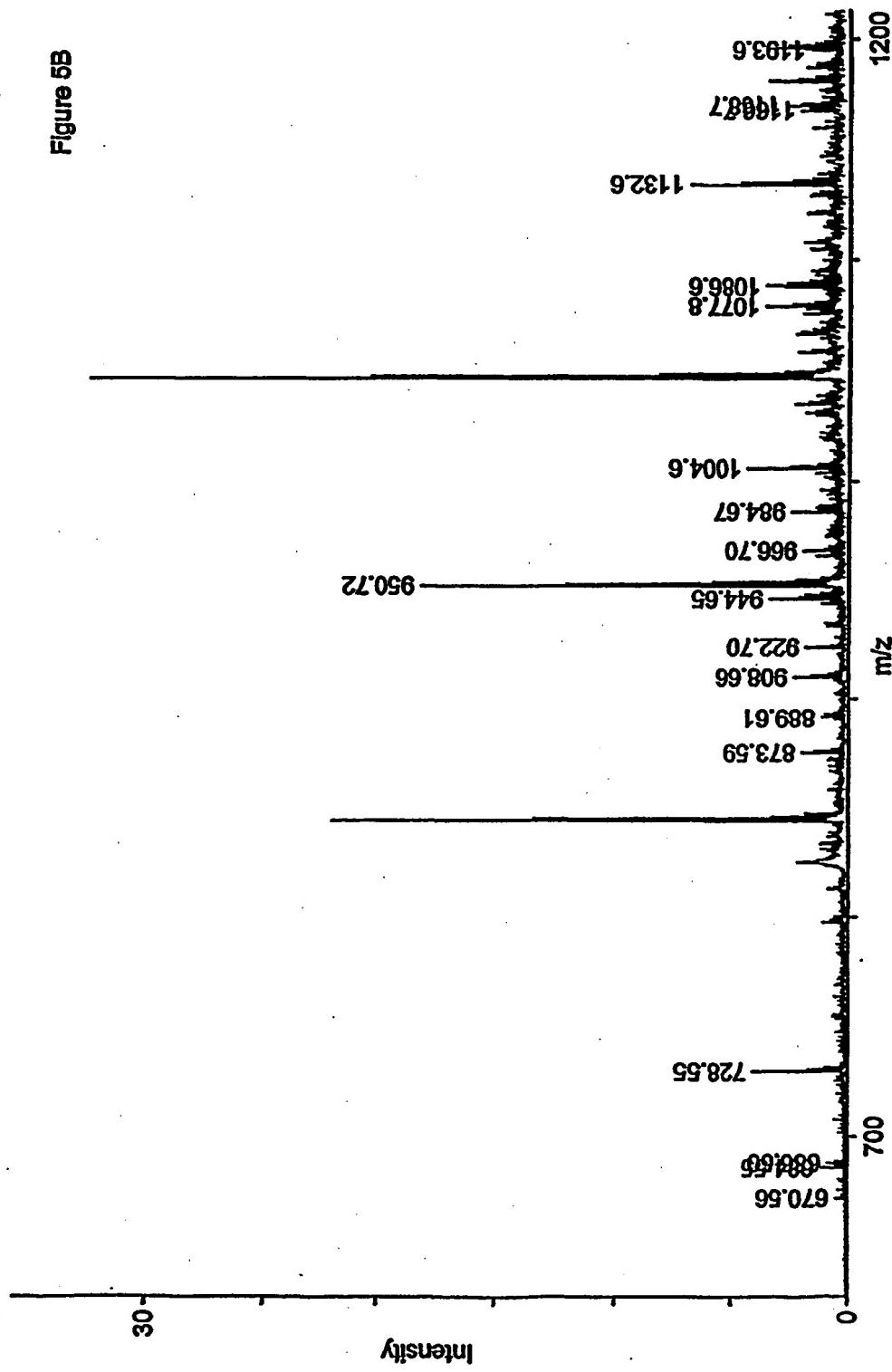
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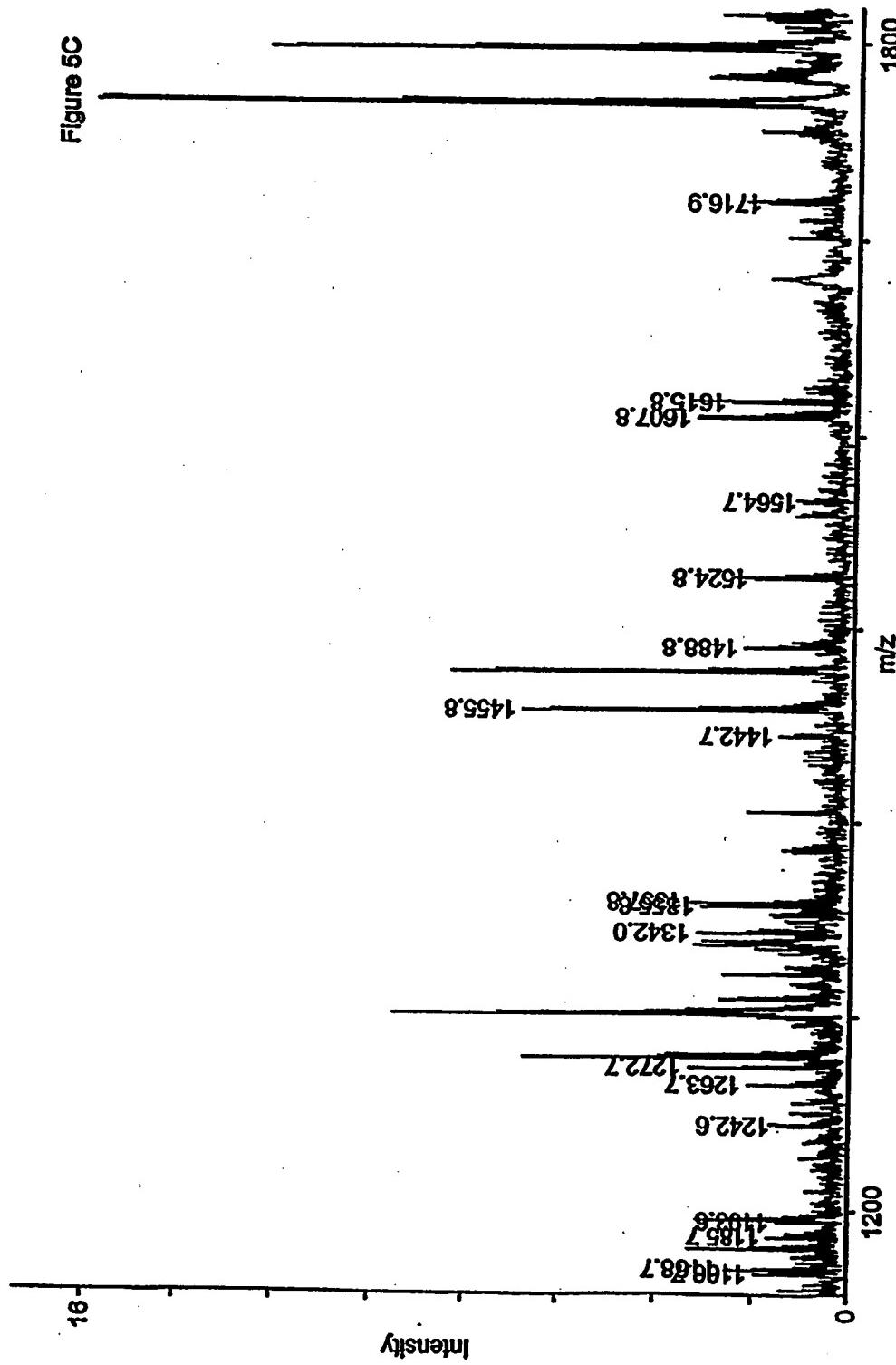
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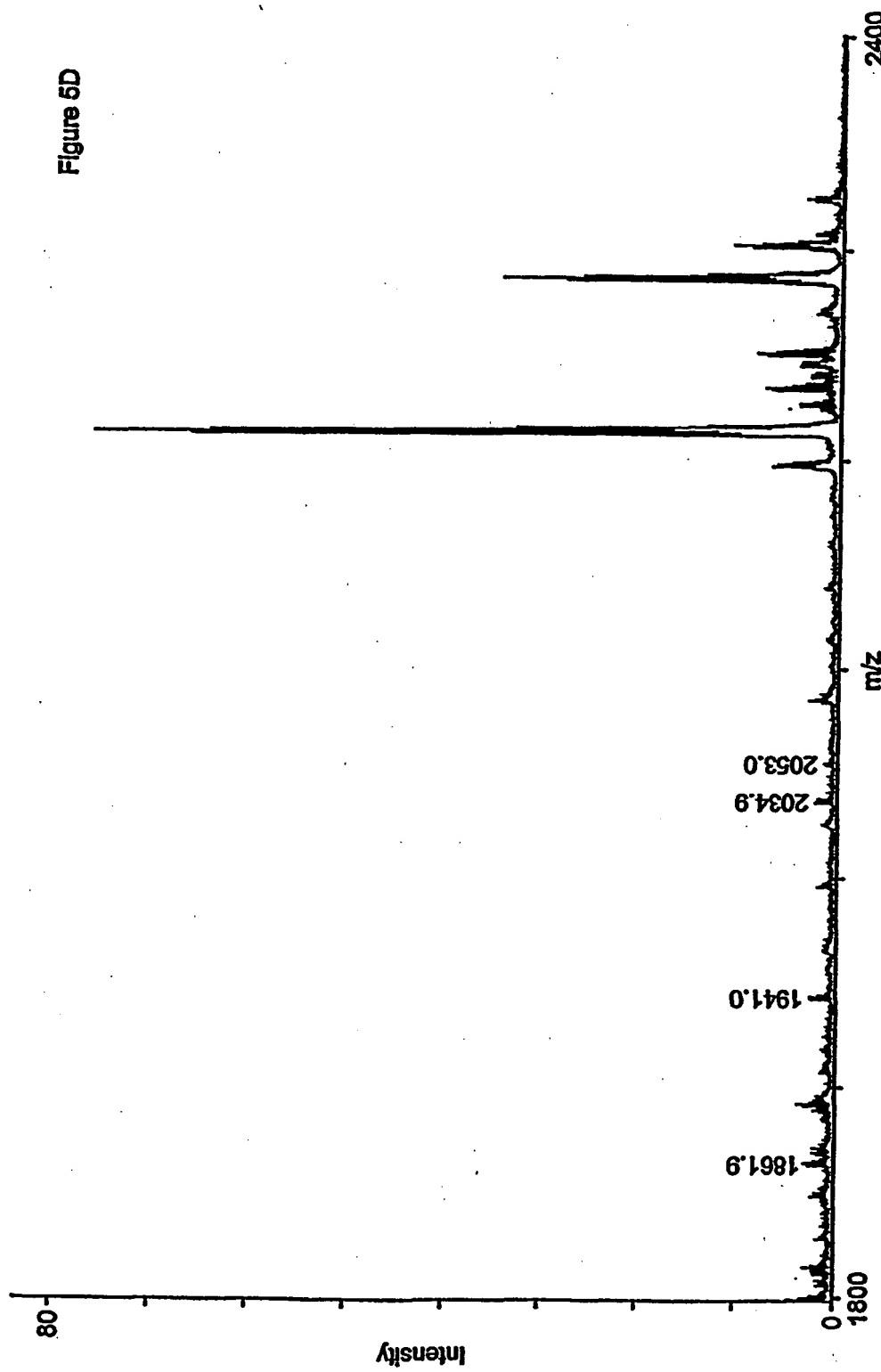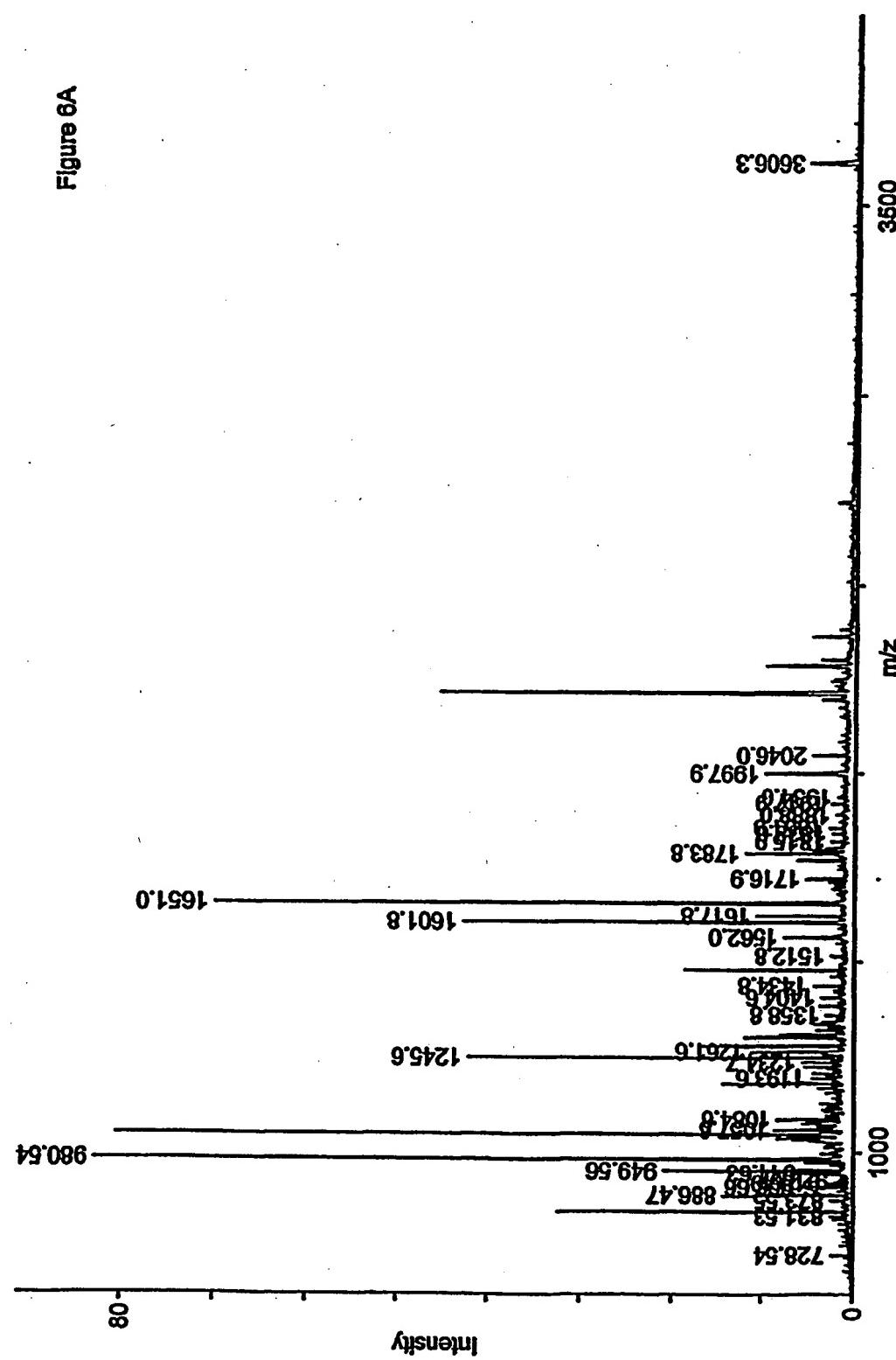
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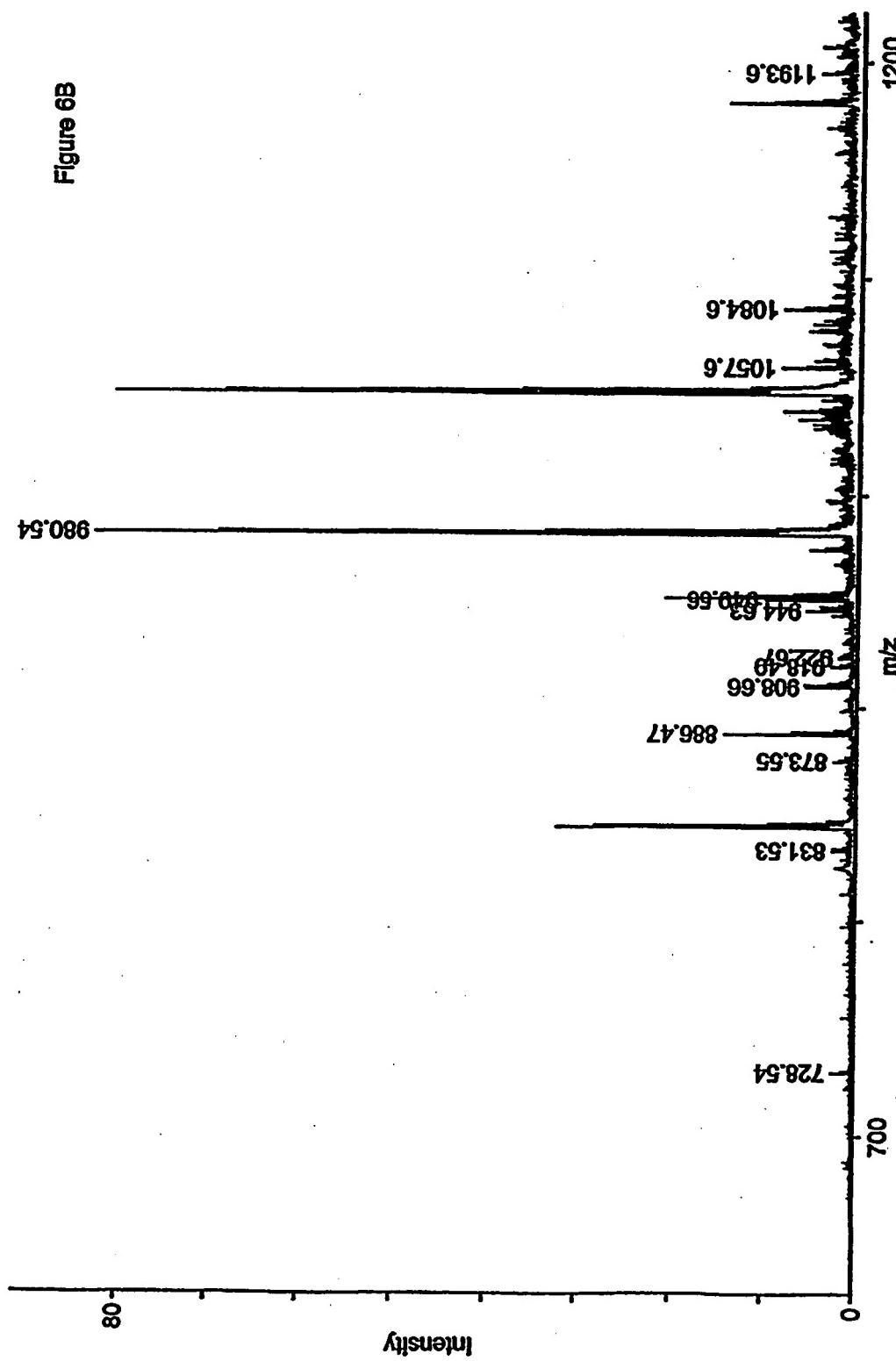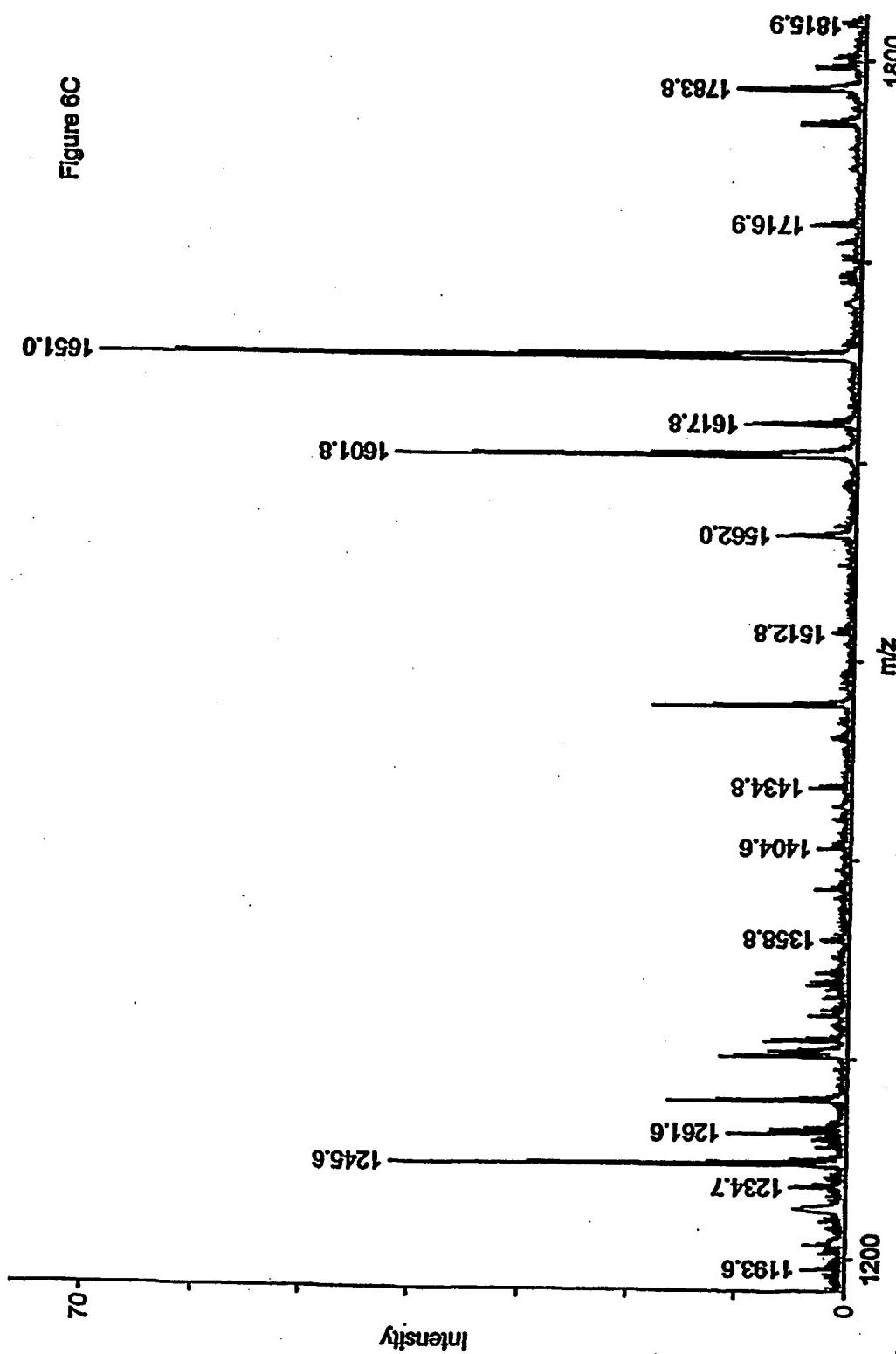
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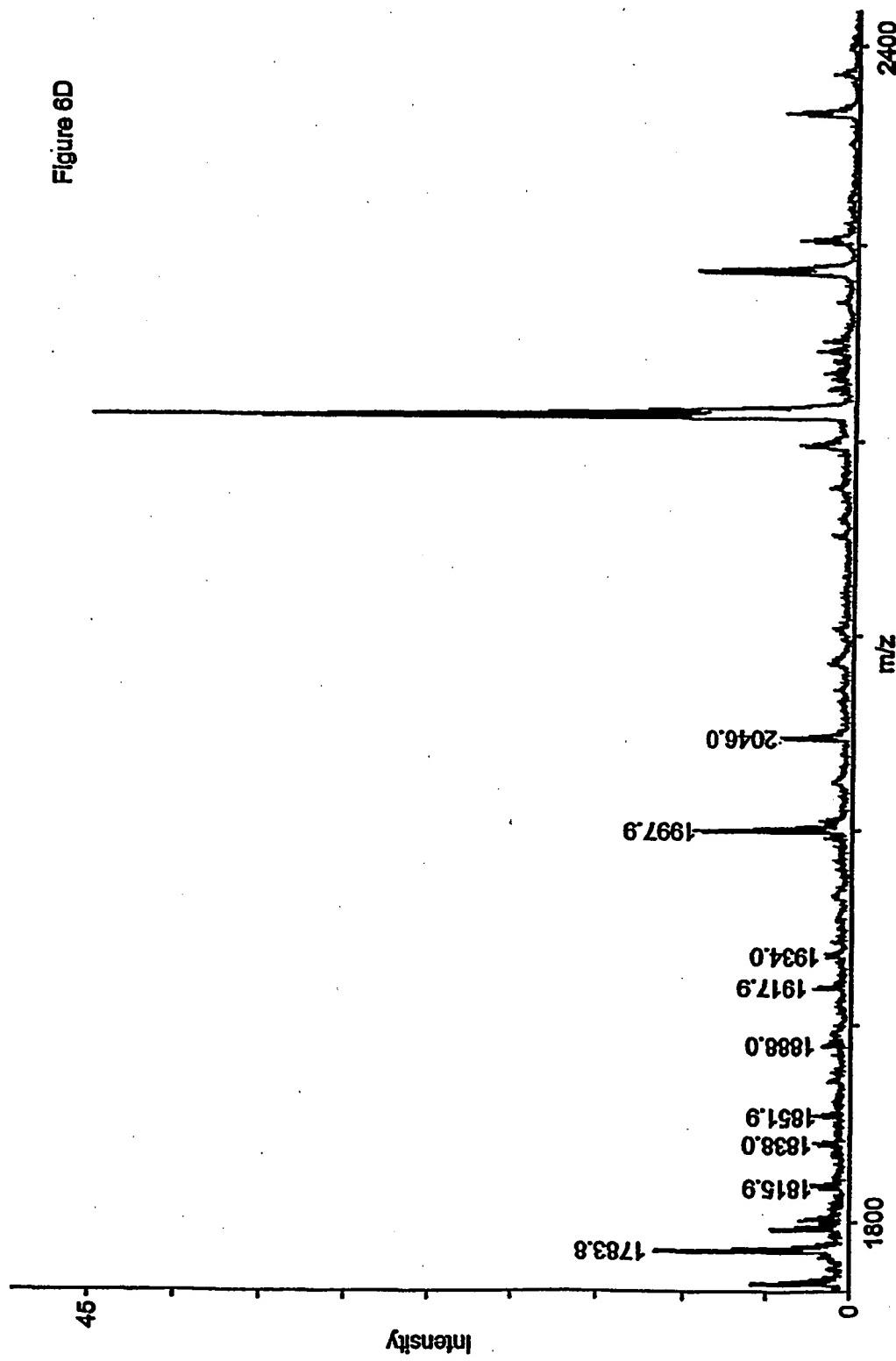
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Figure 6D

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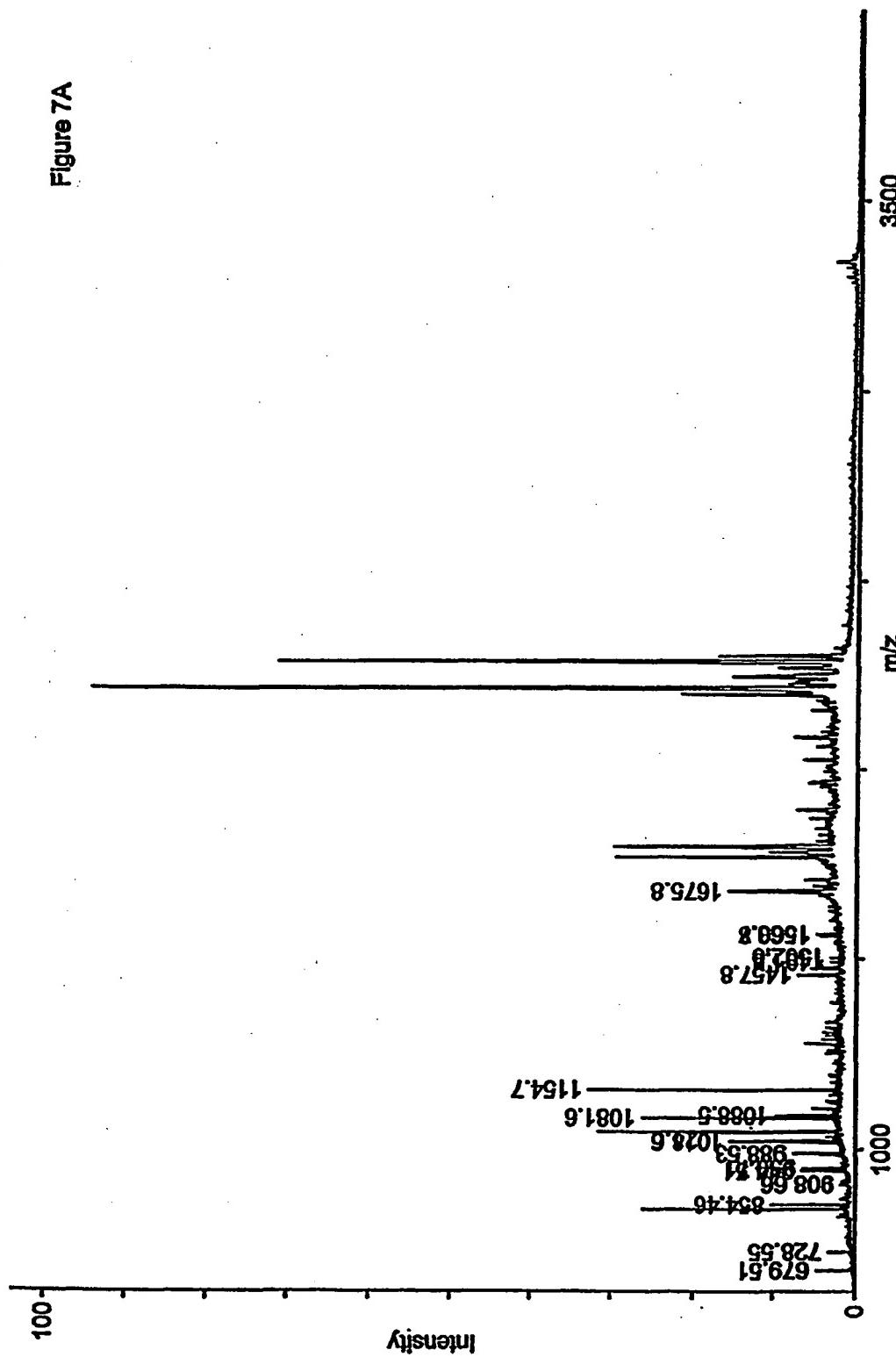
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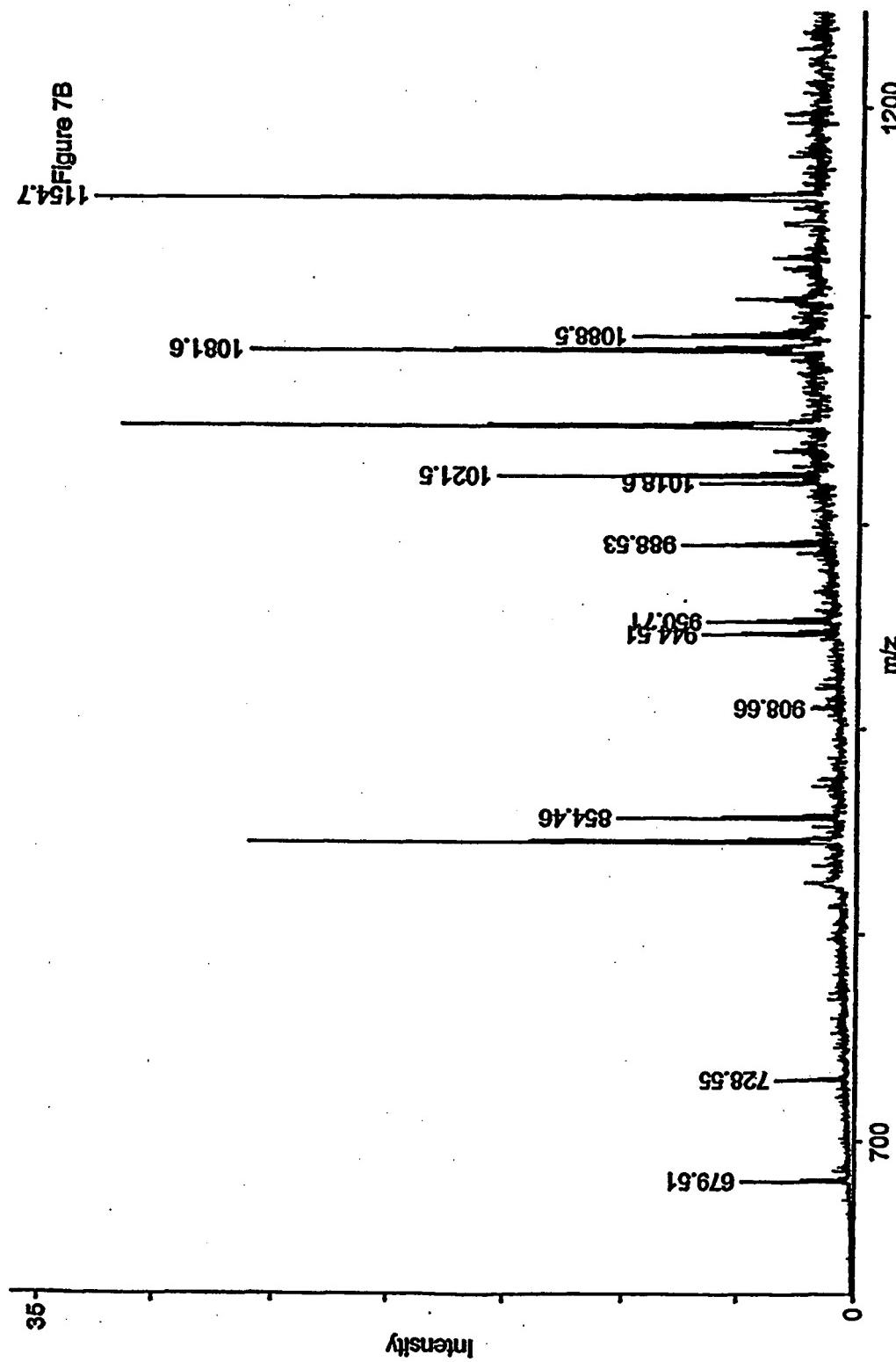
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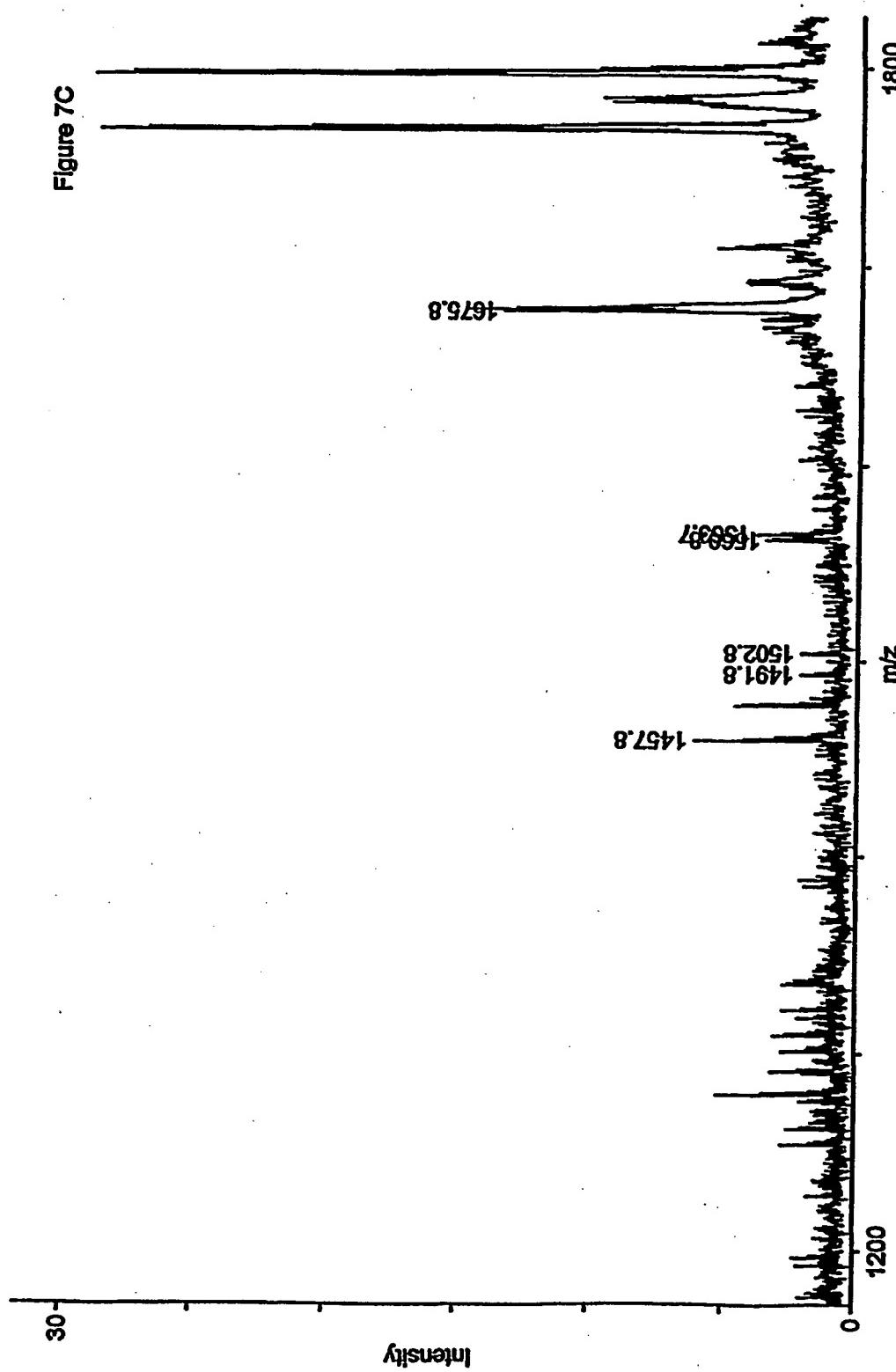
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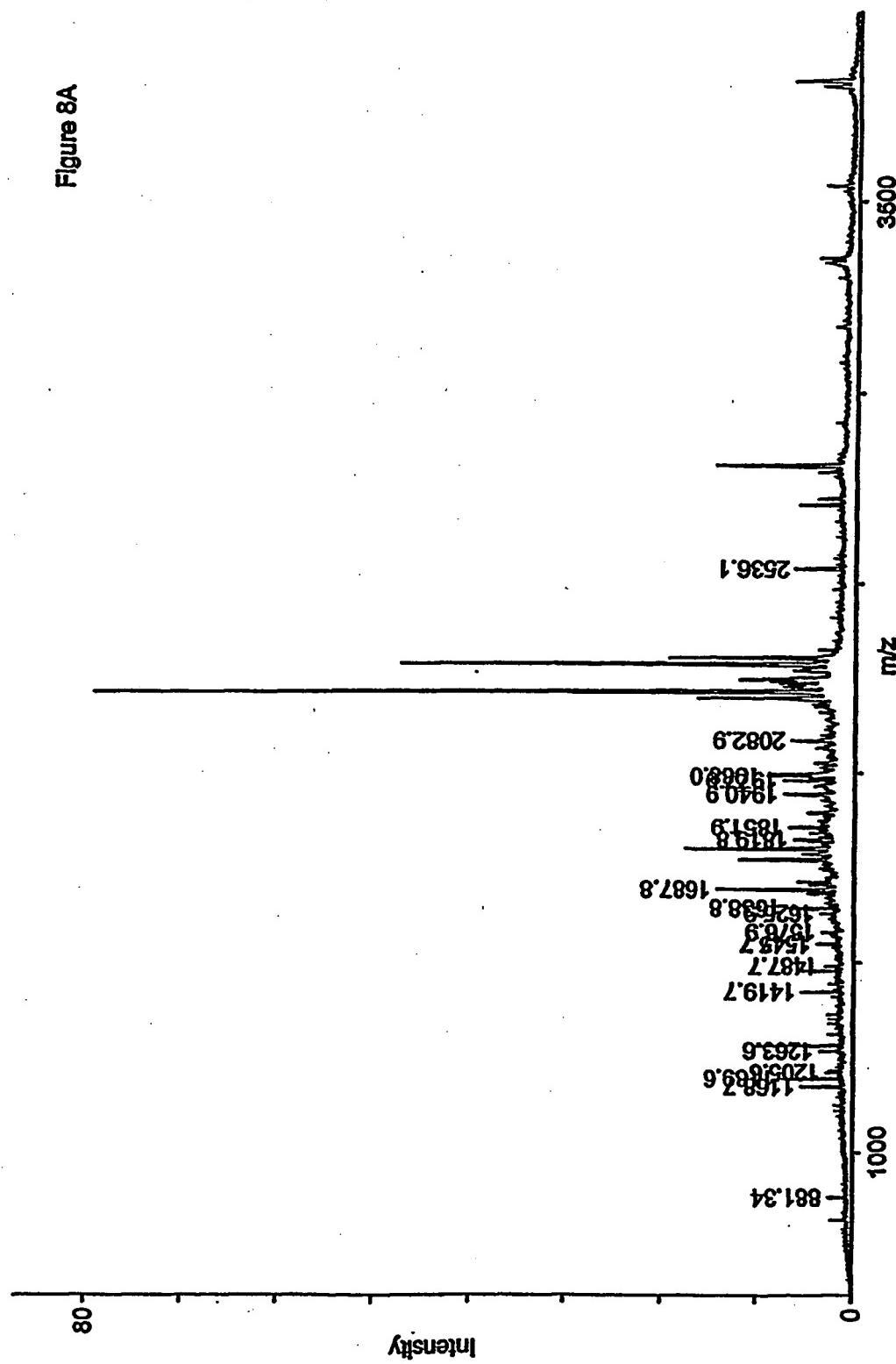
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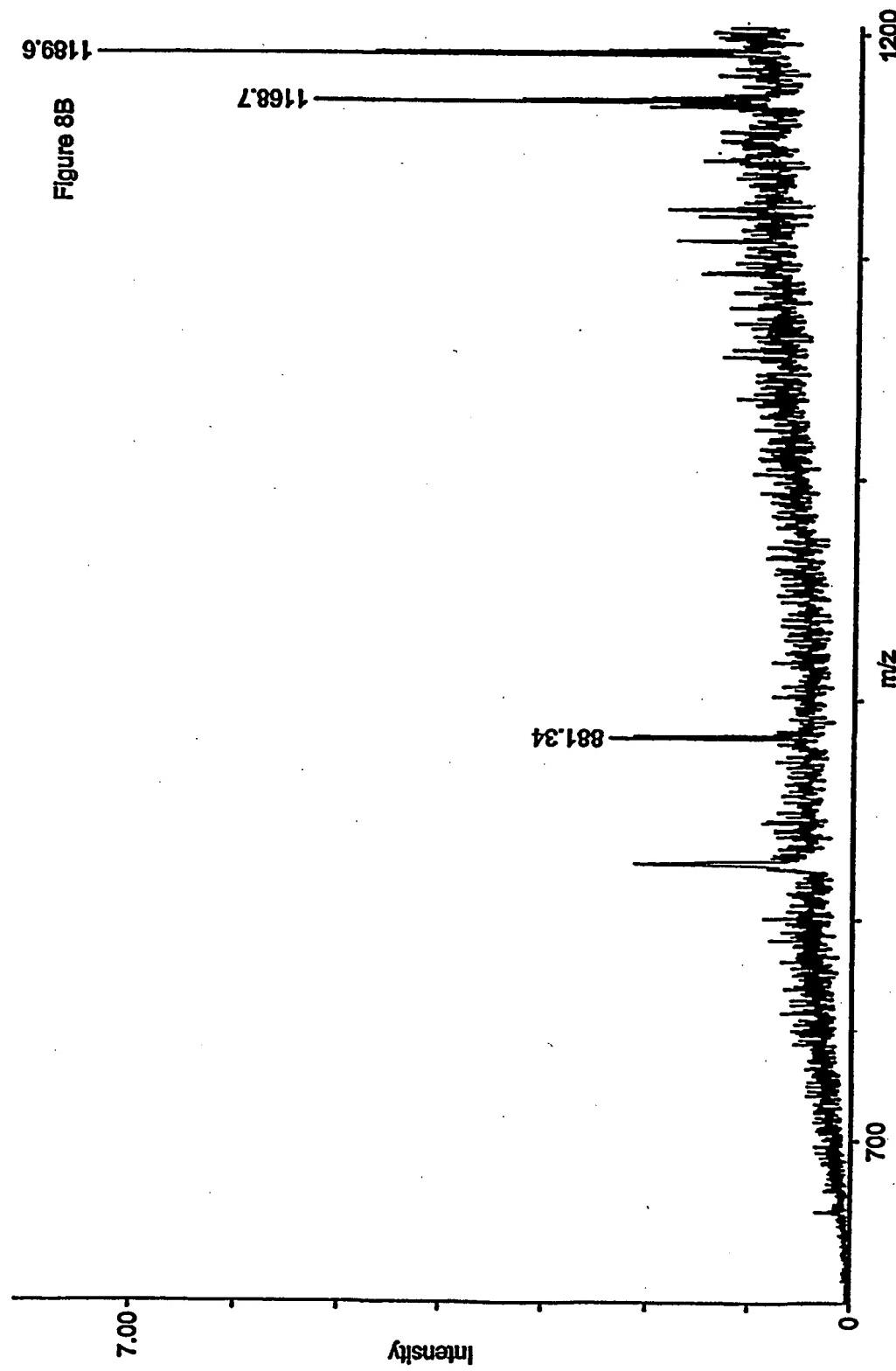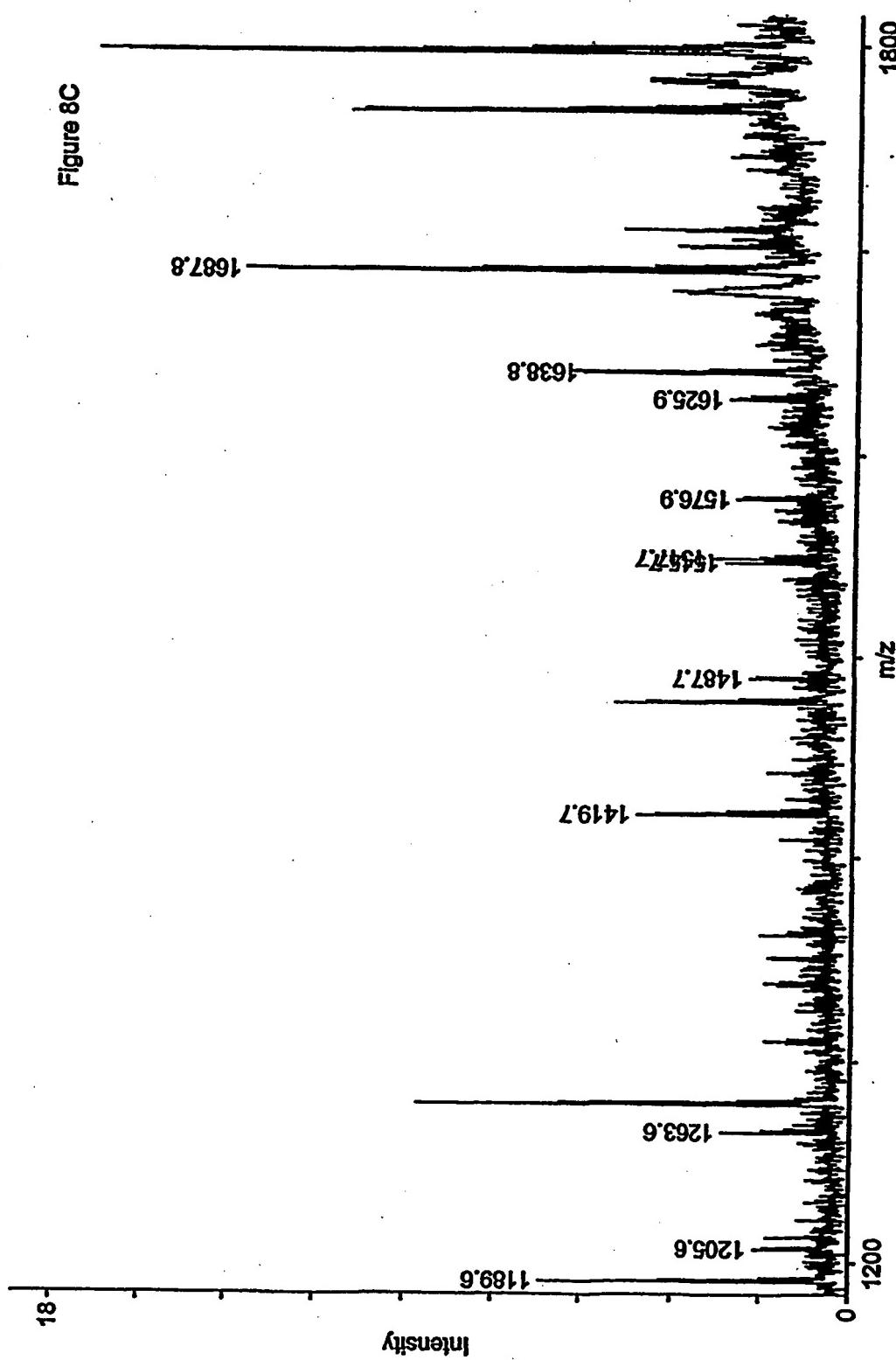
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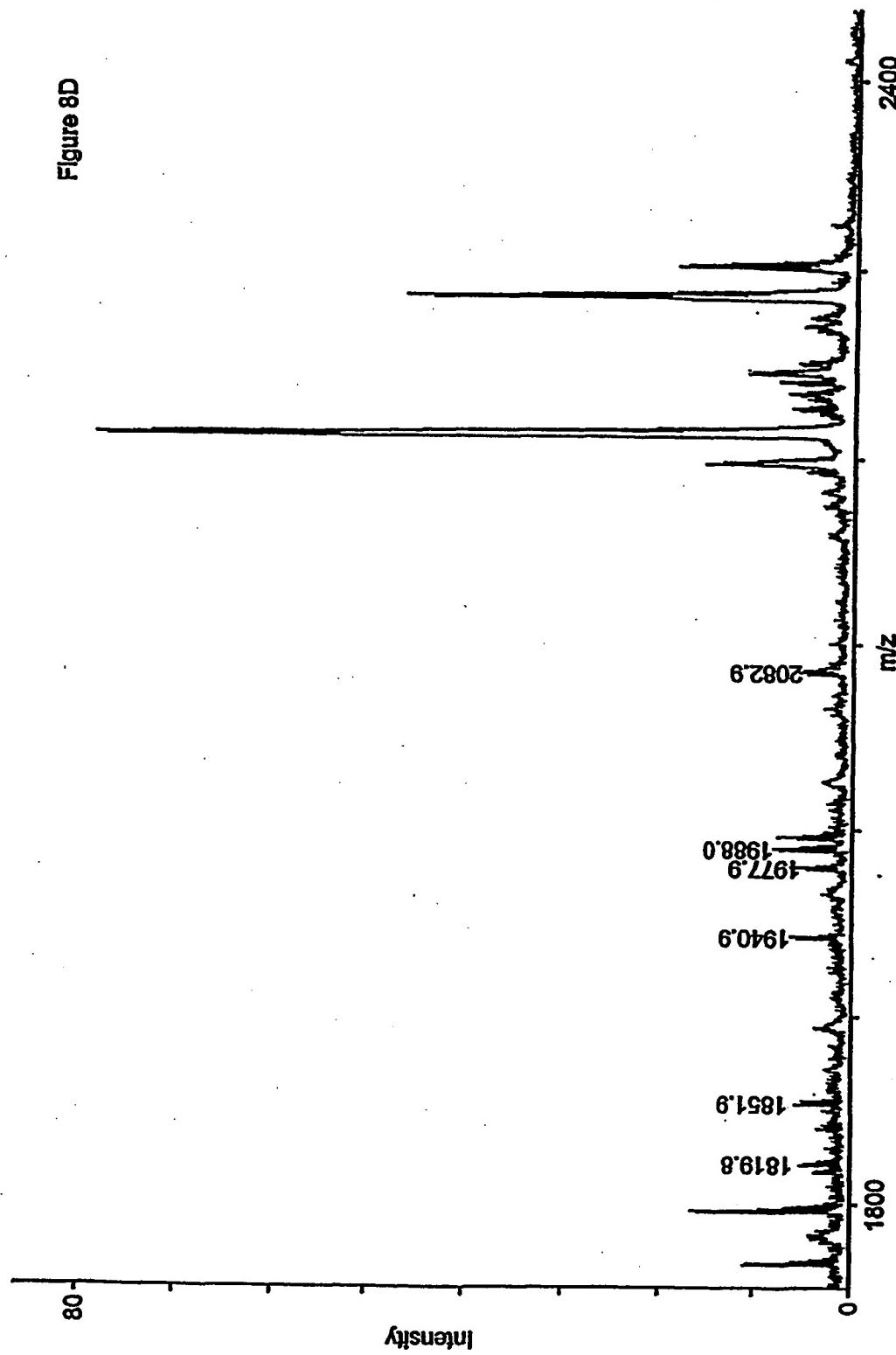
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Figure 8E

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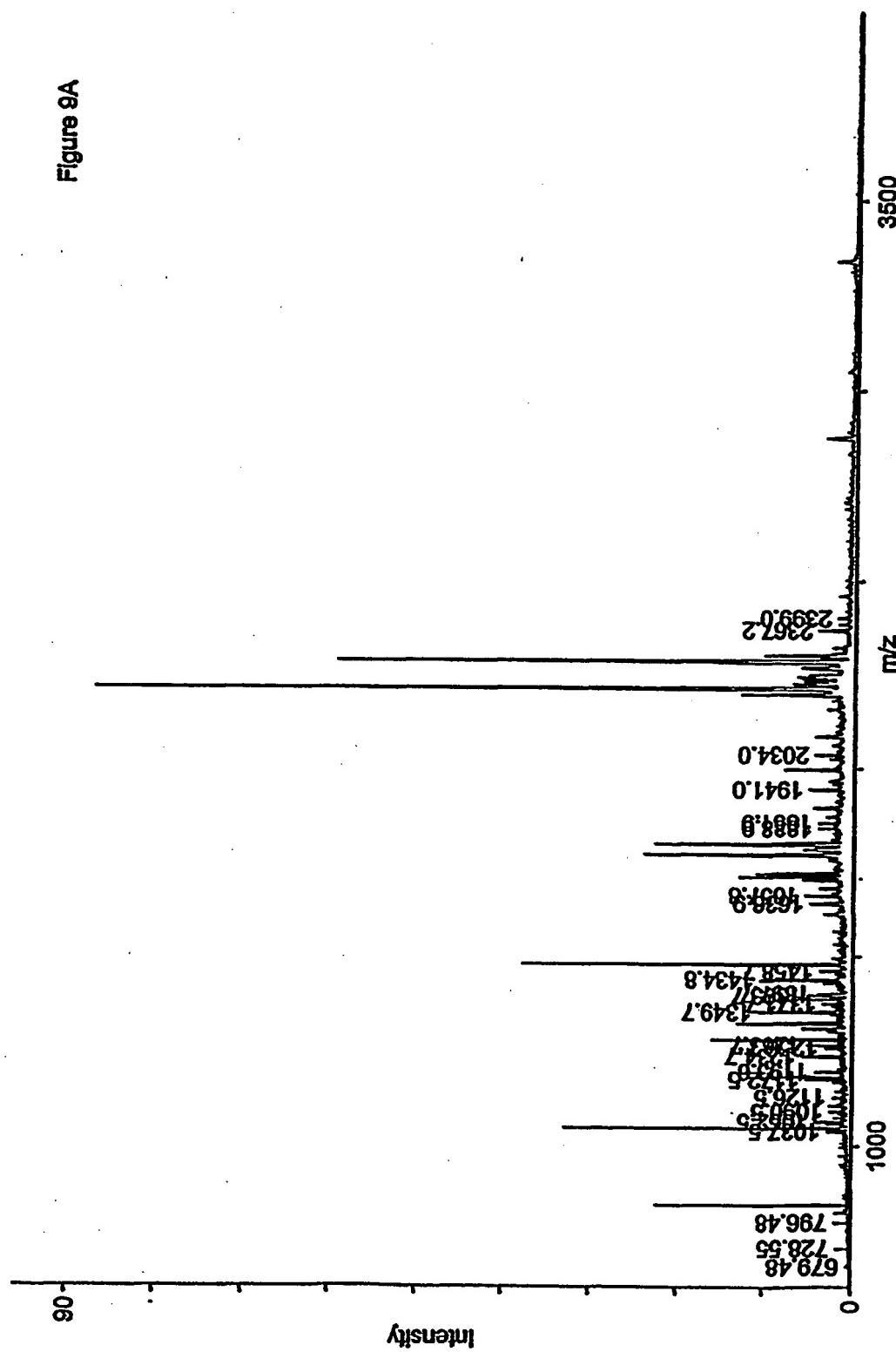
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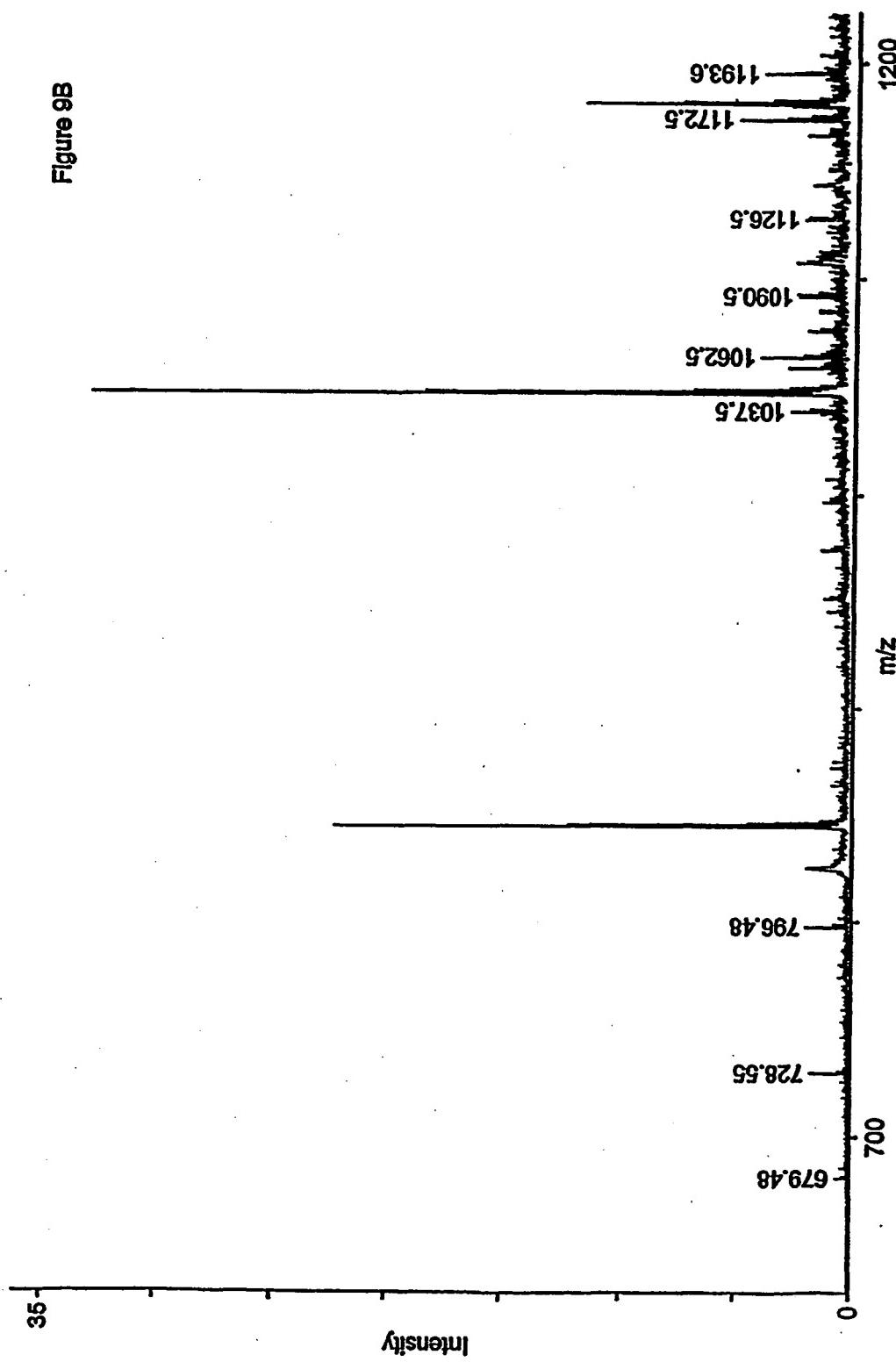
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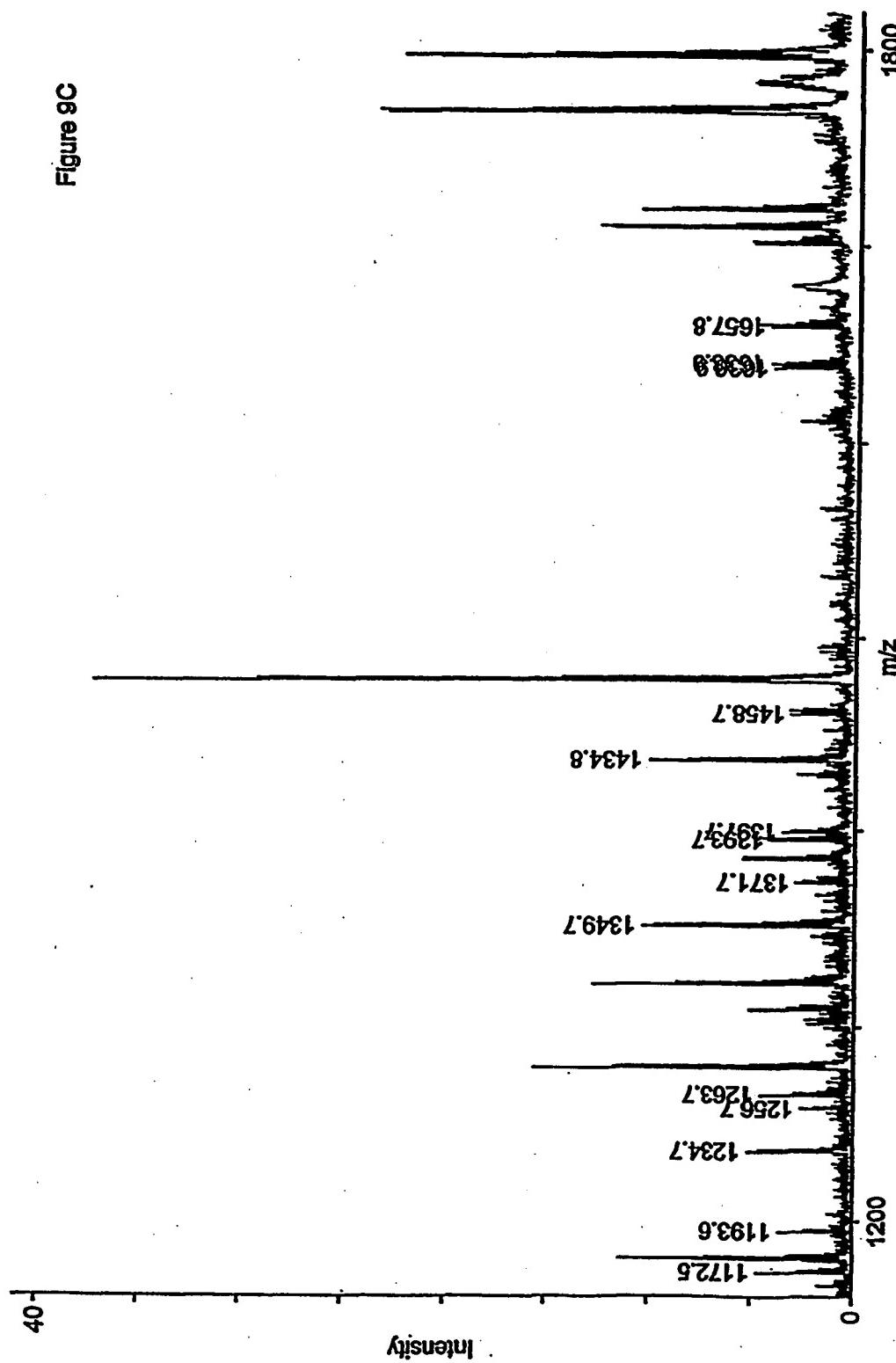
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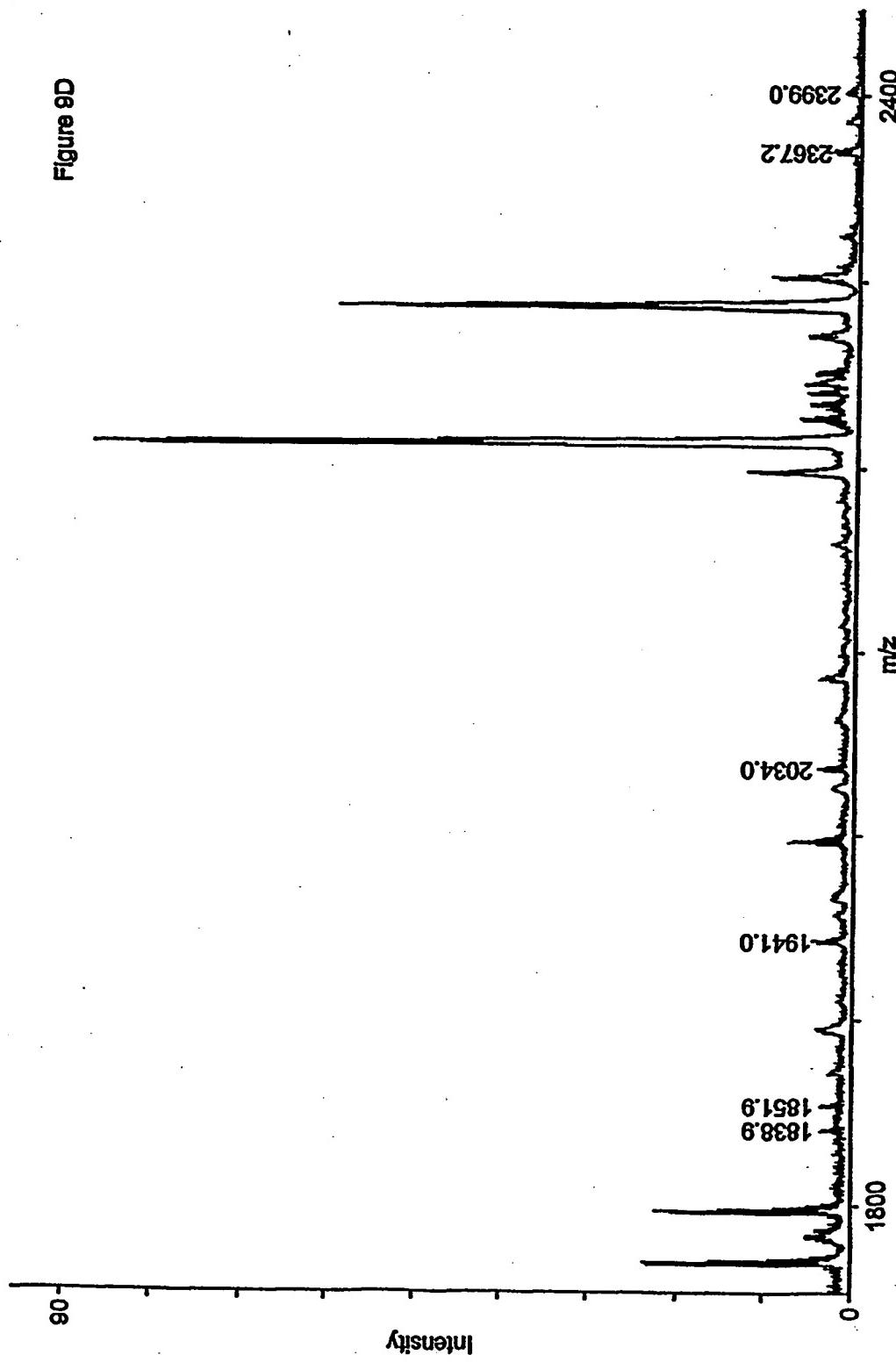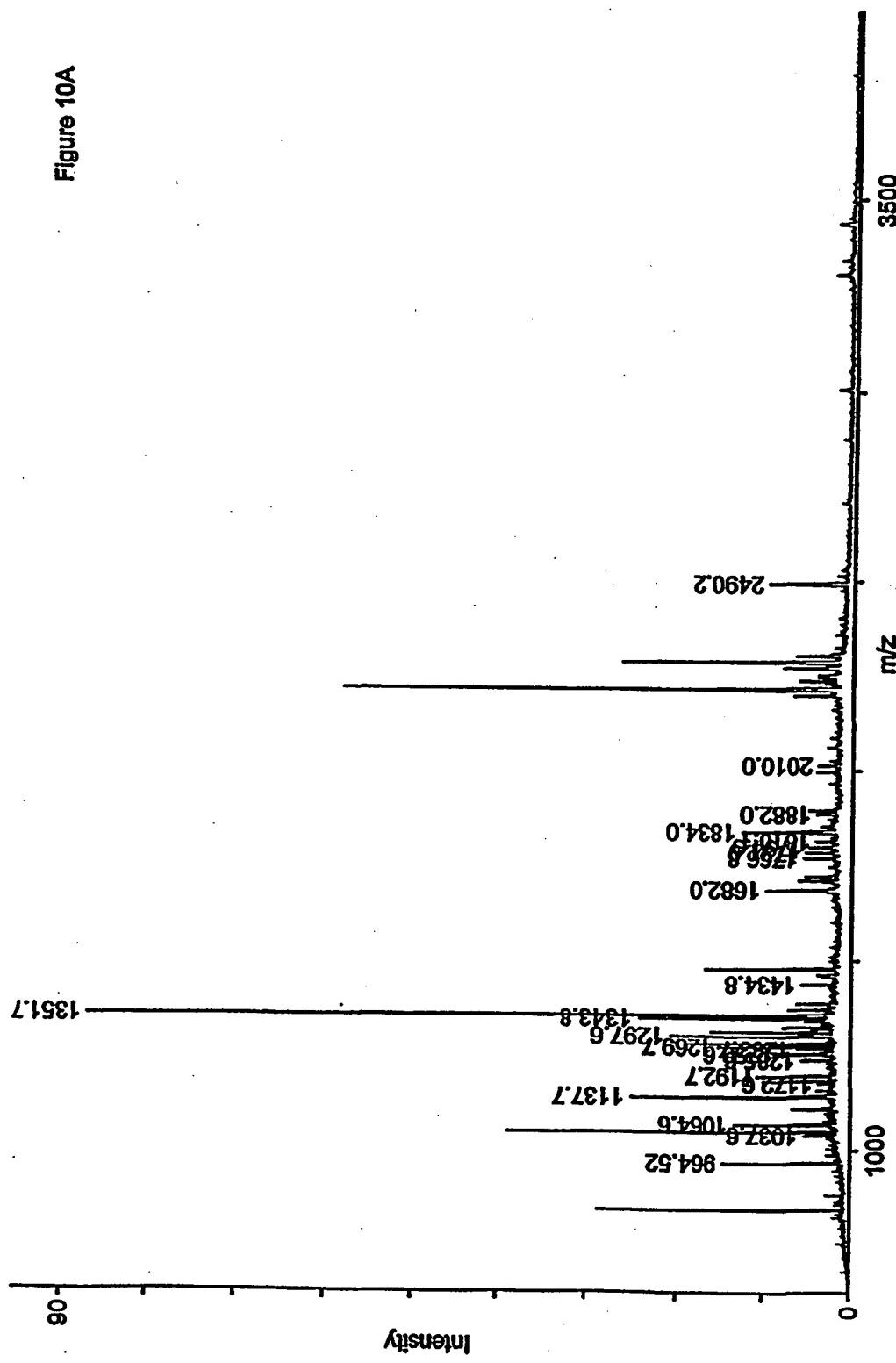
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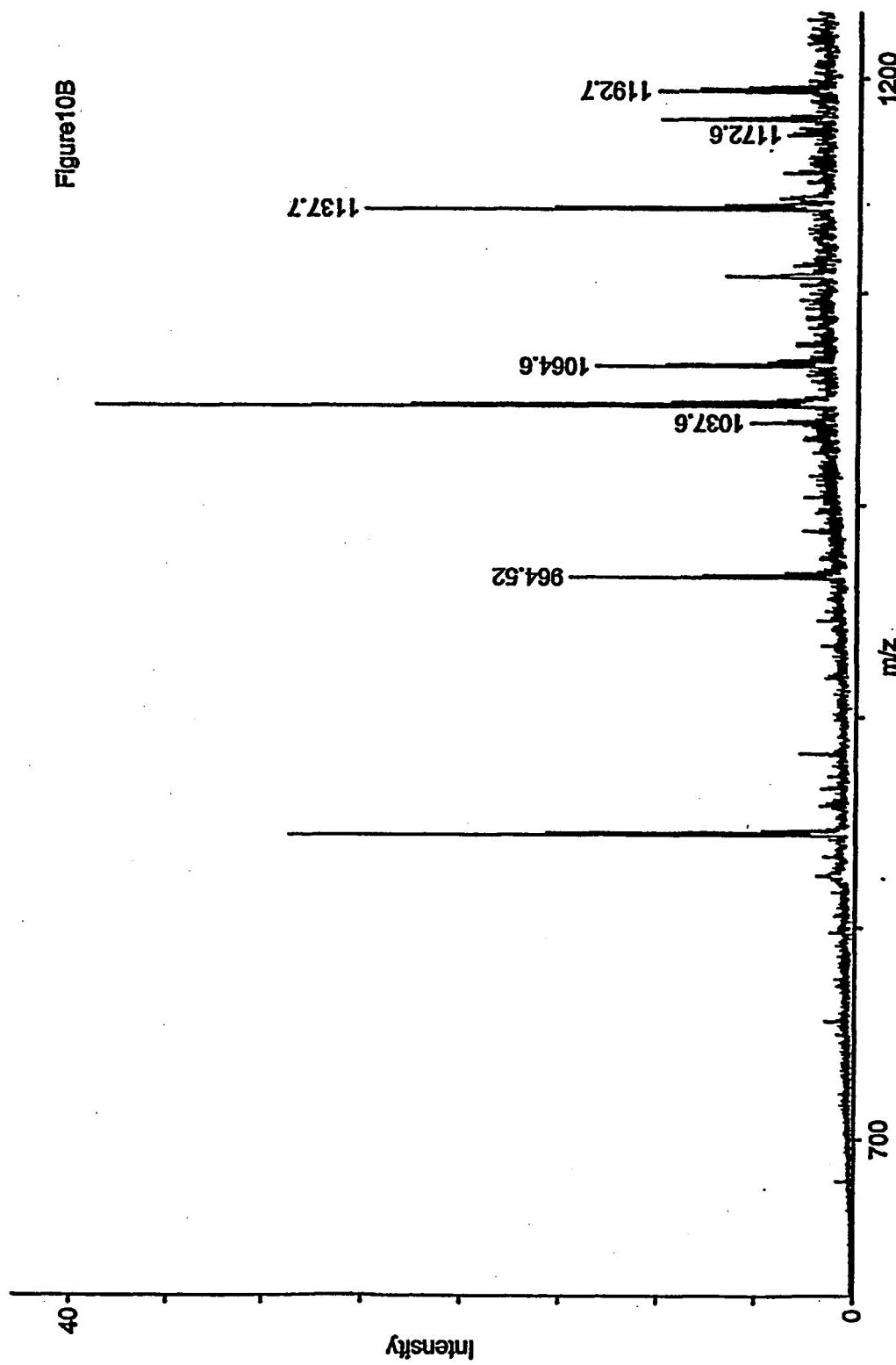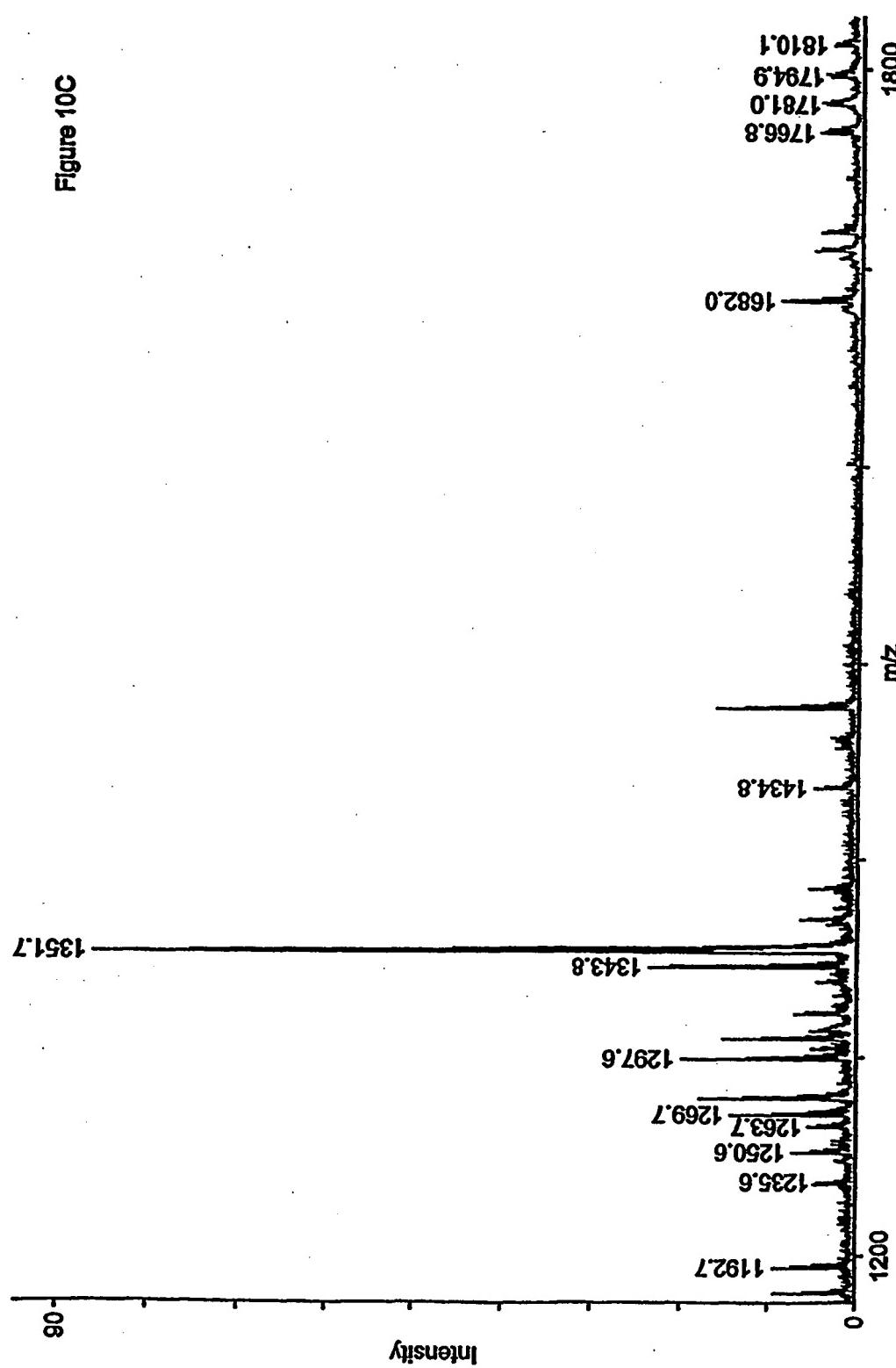
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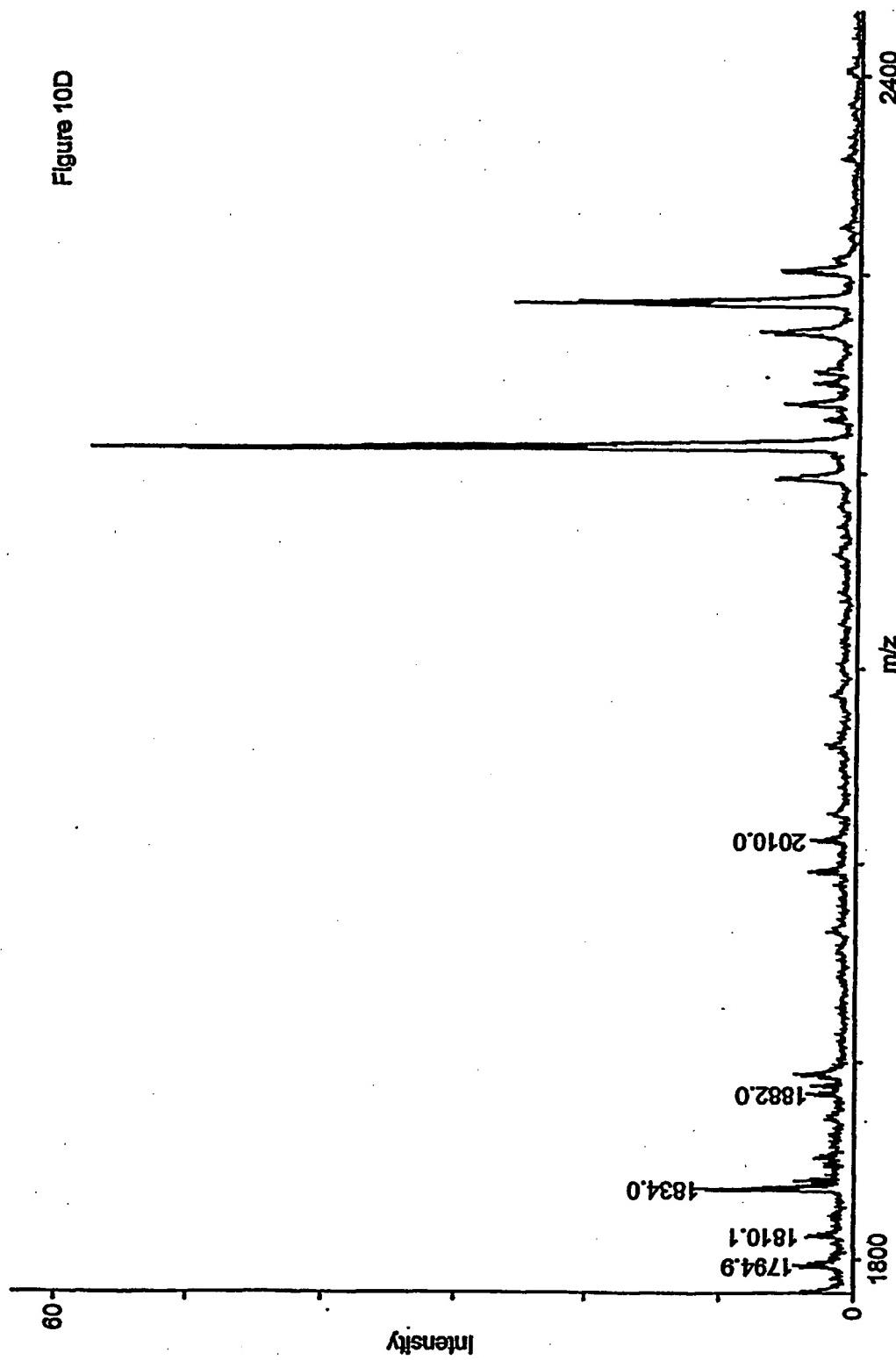
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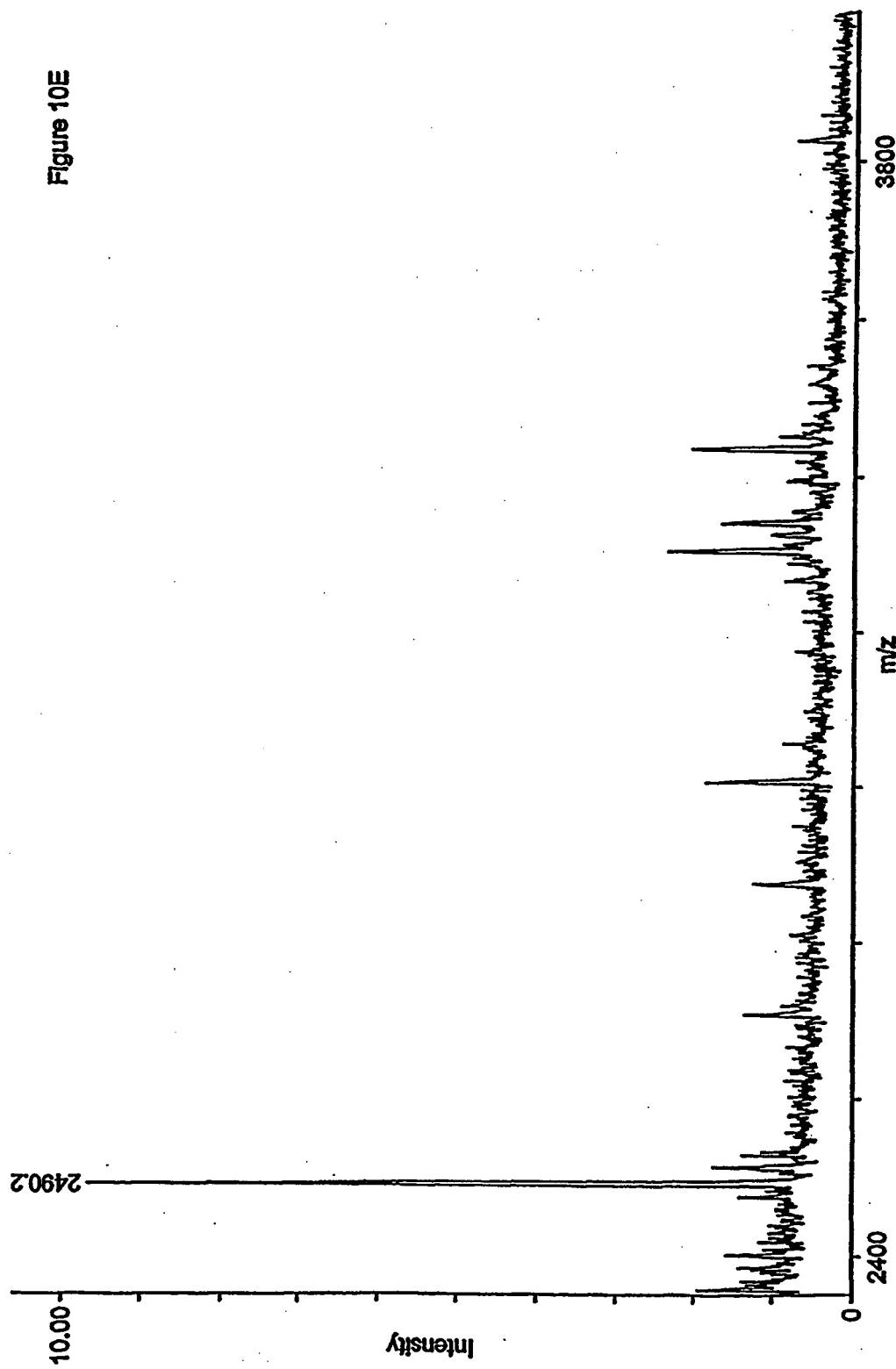
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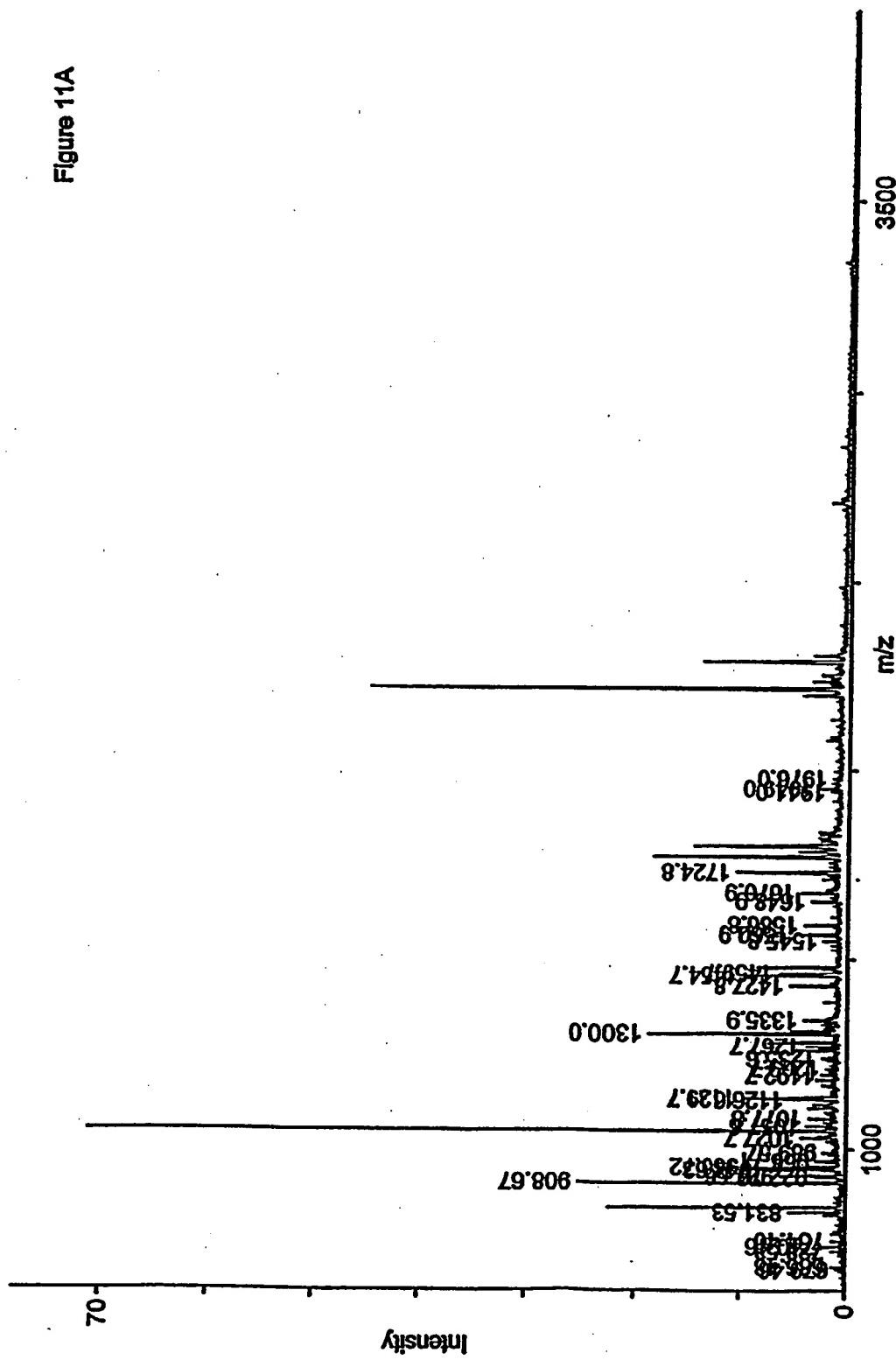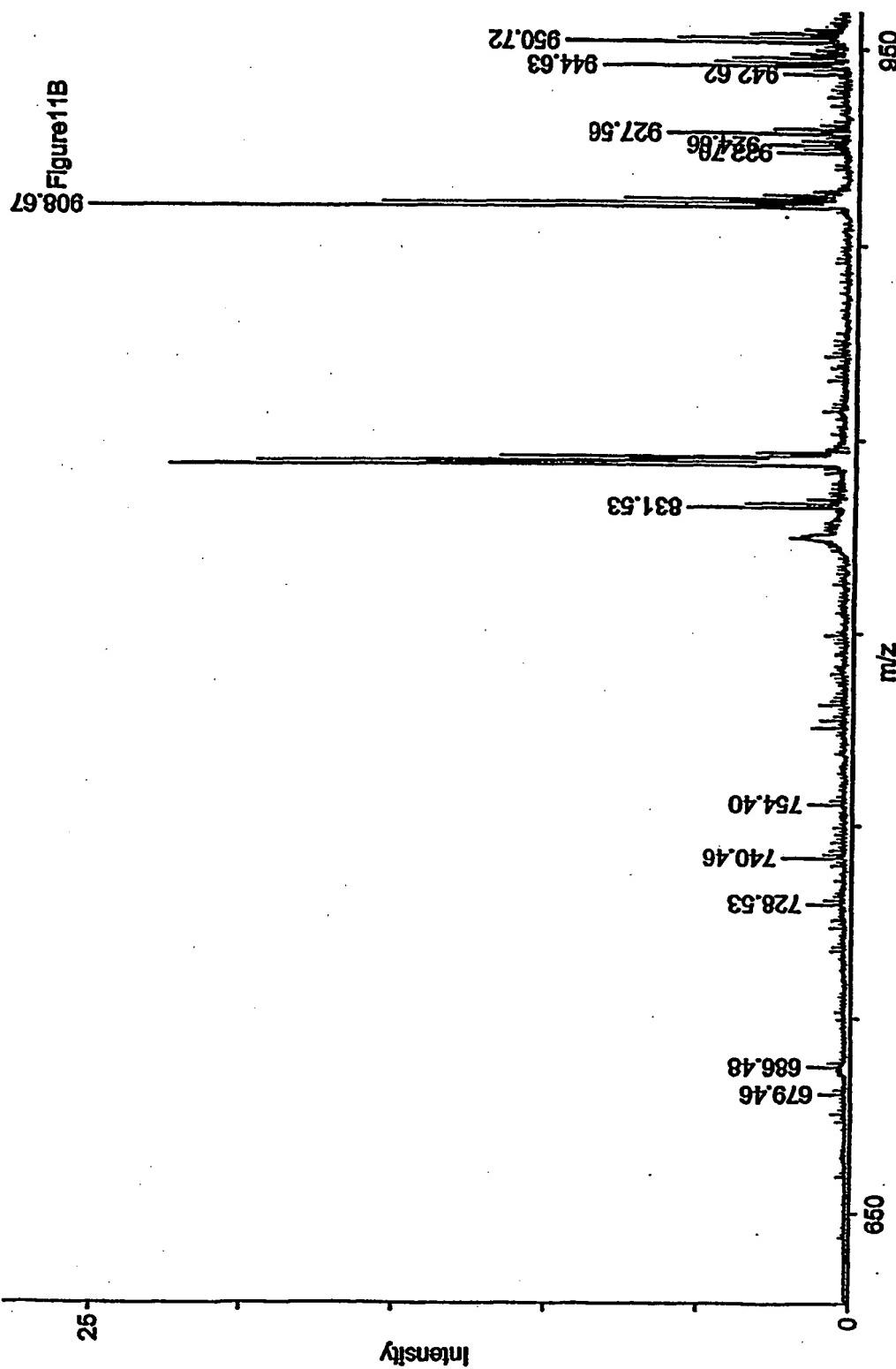
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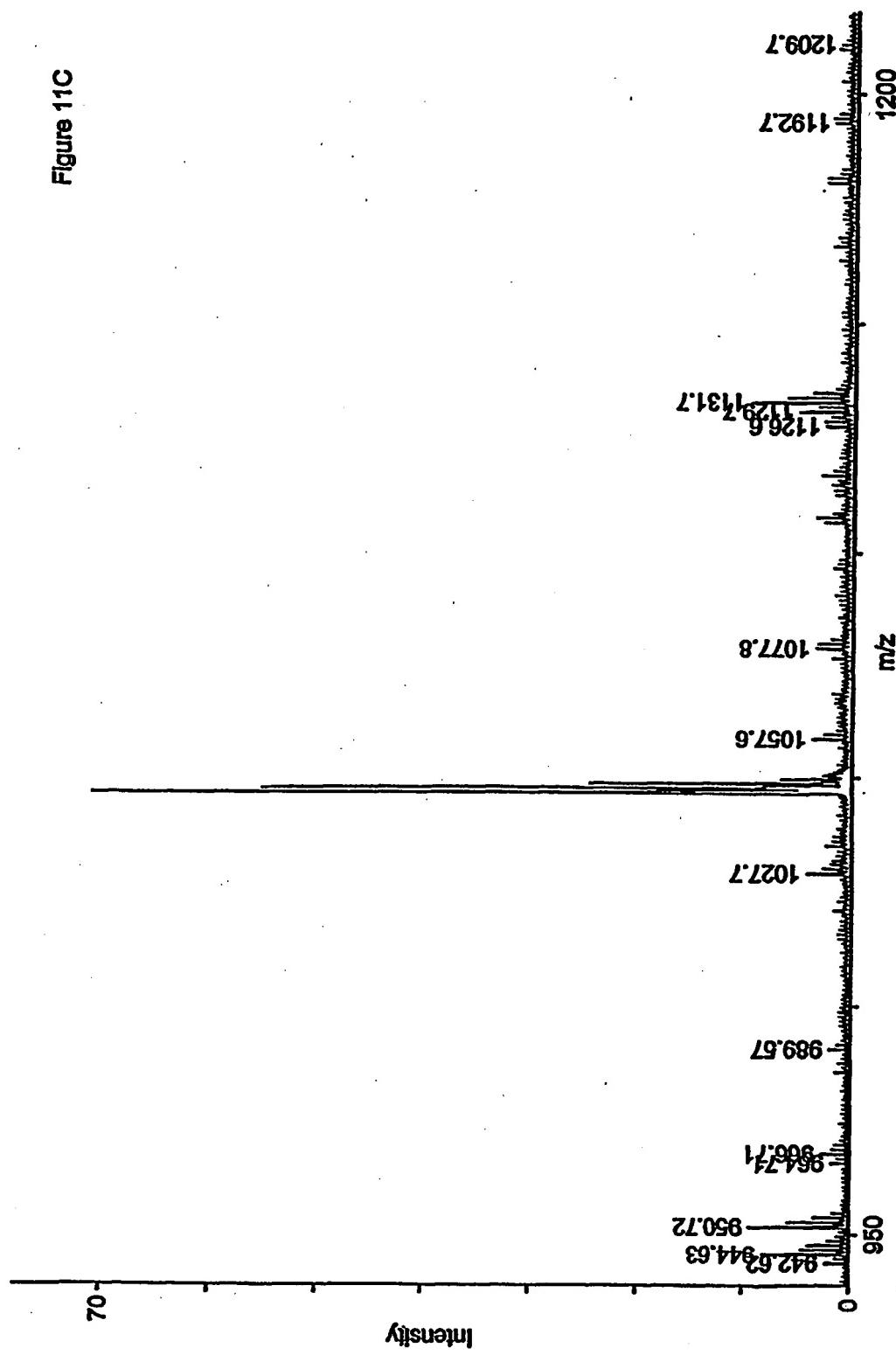
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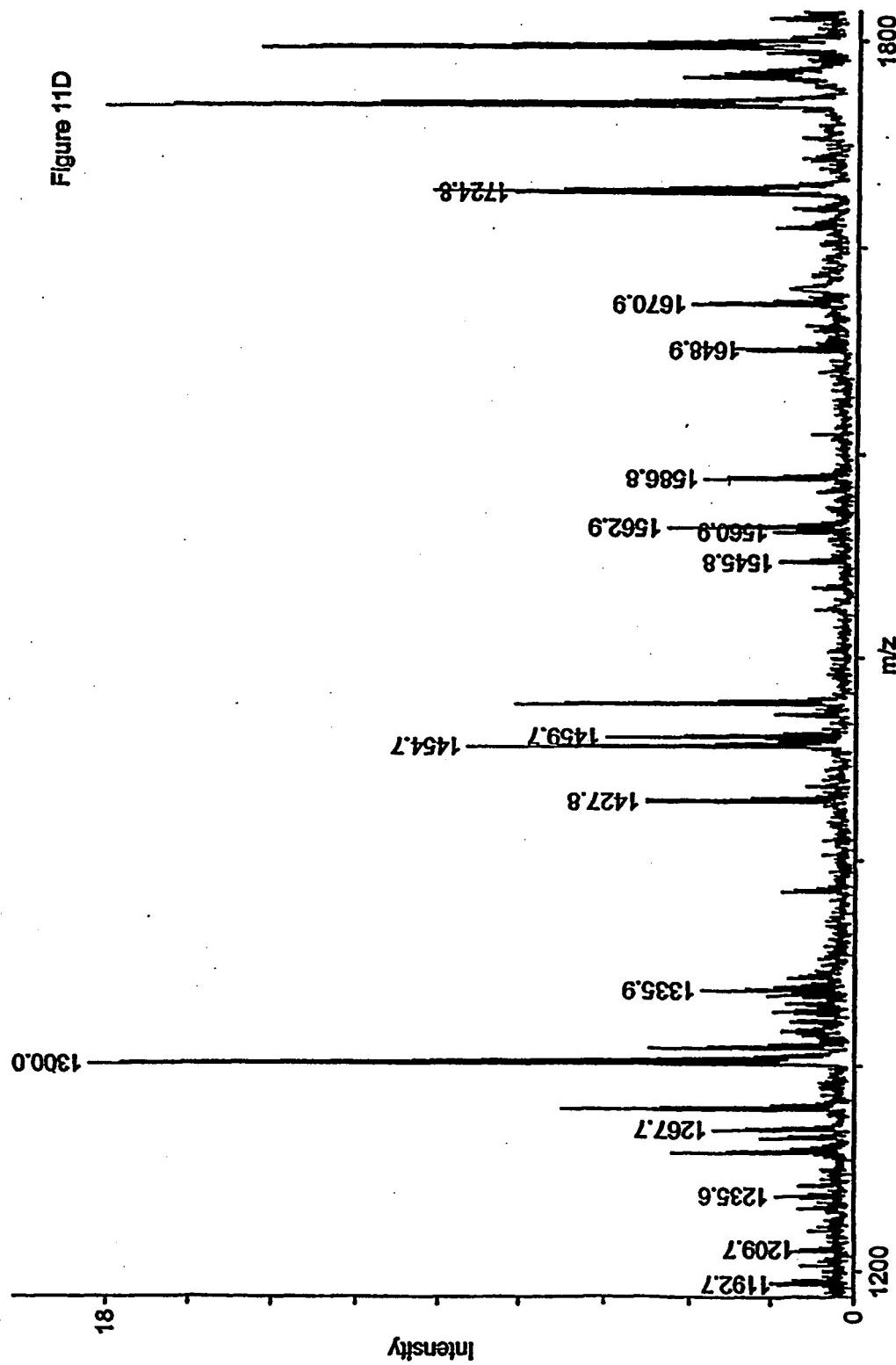
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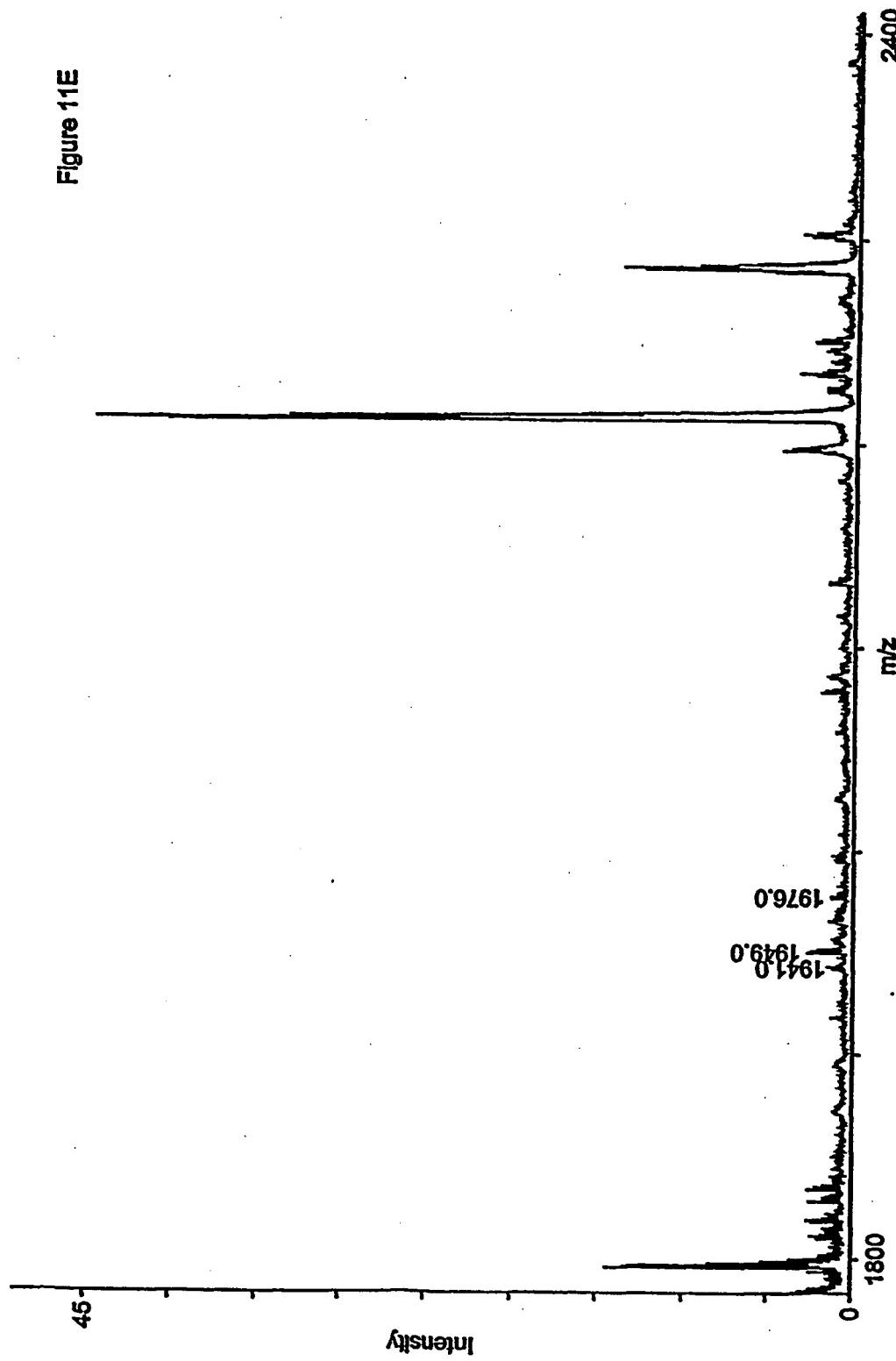
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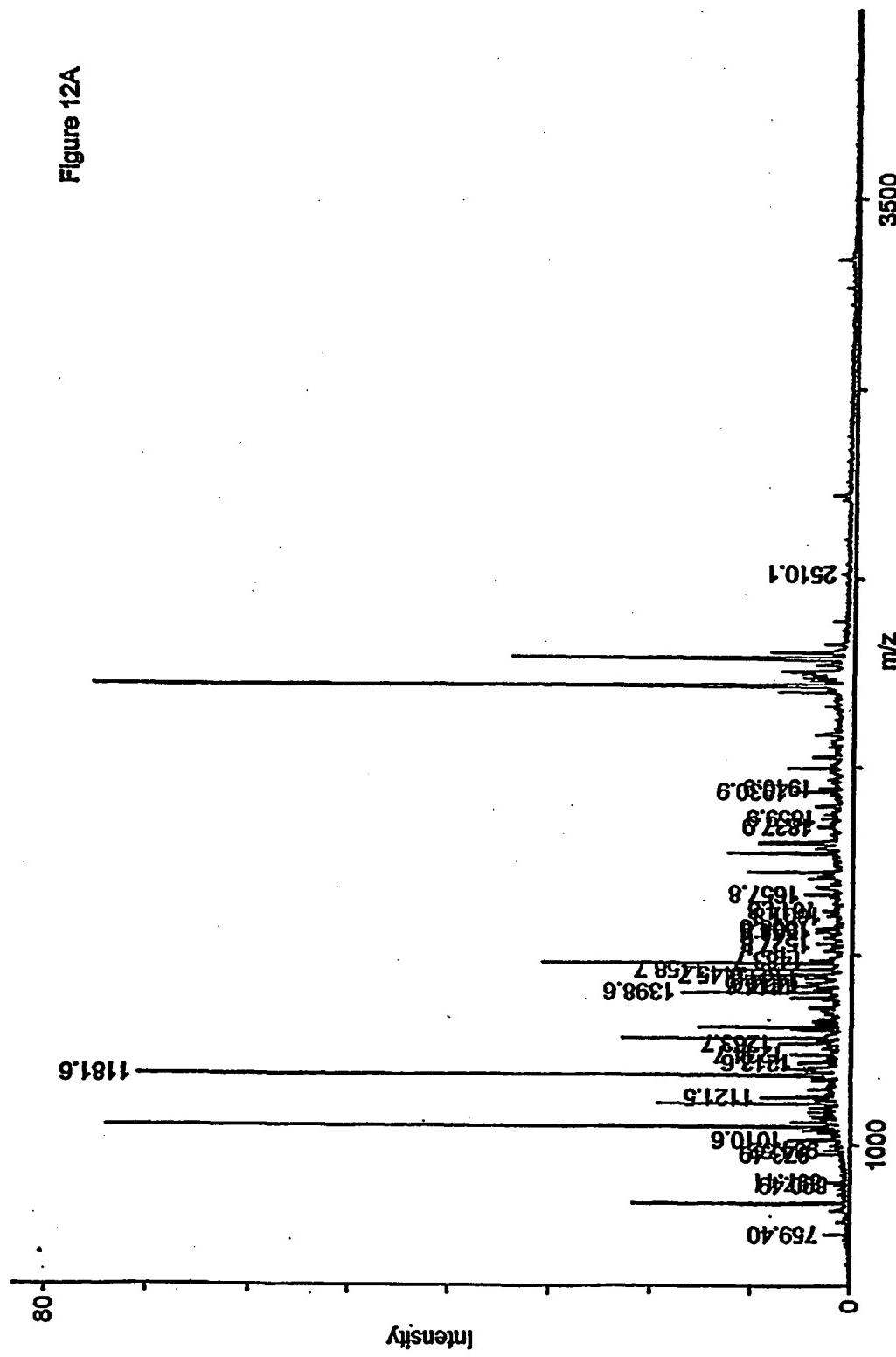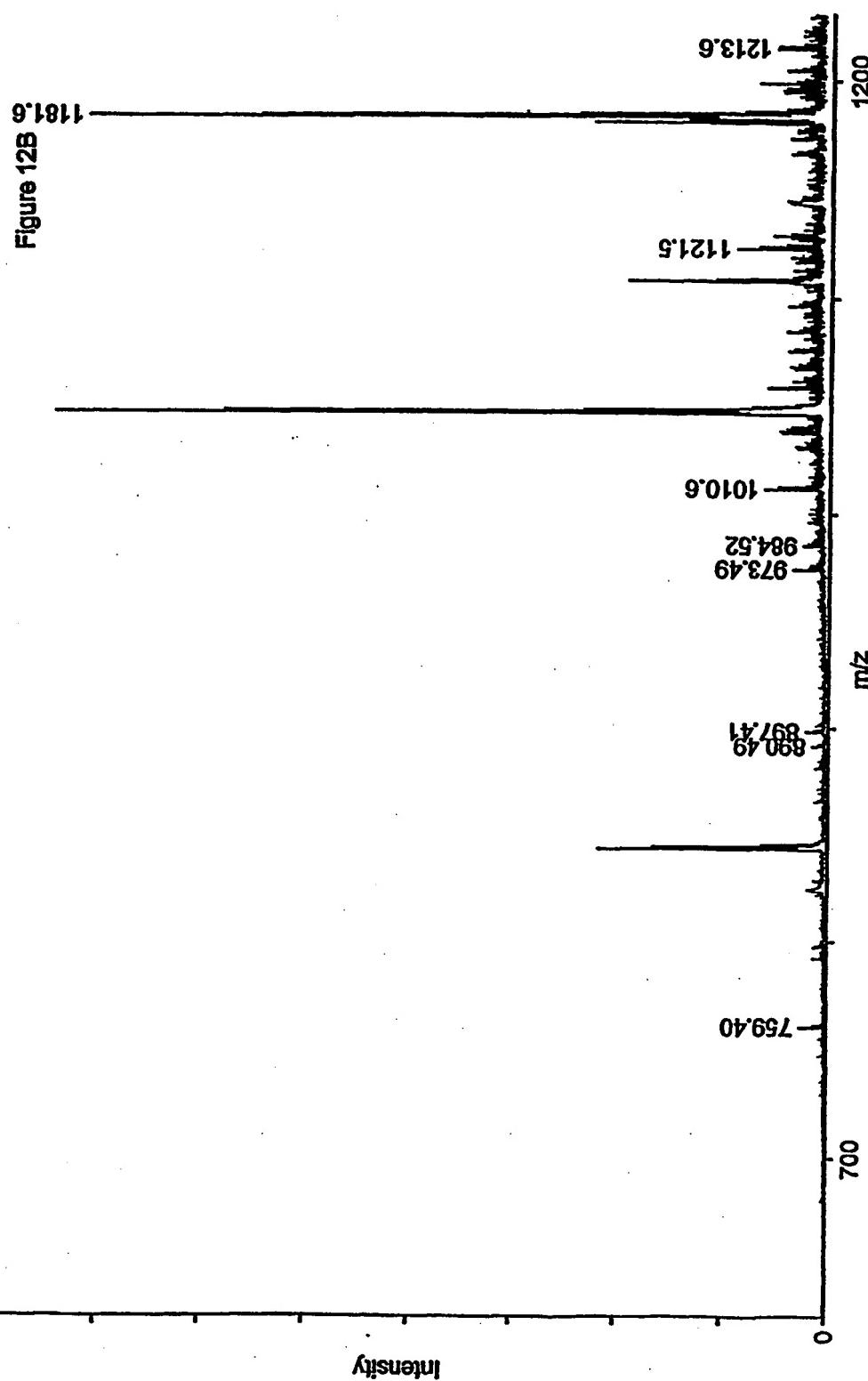
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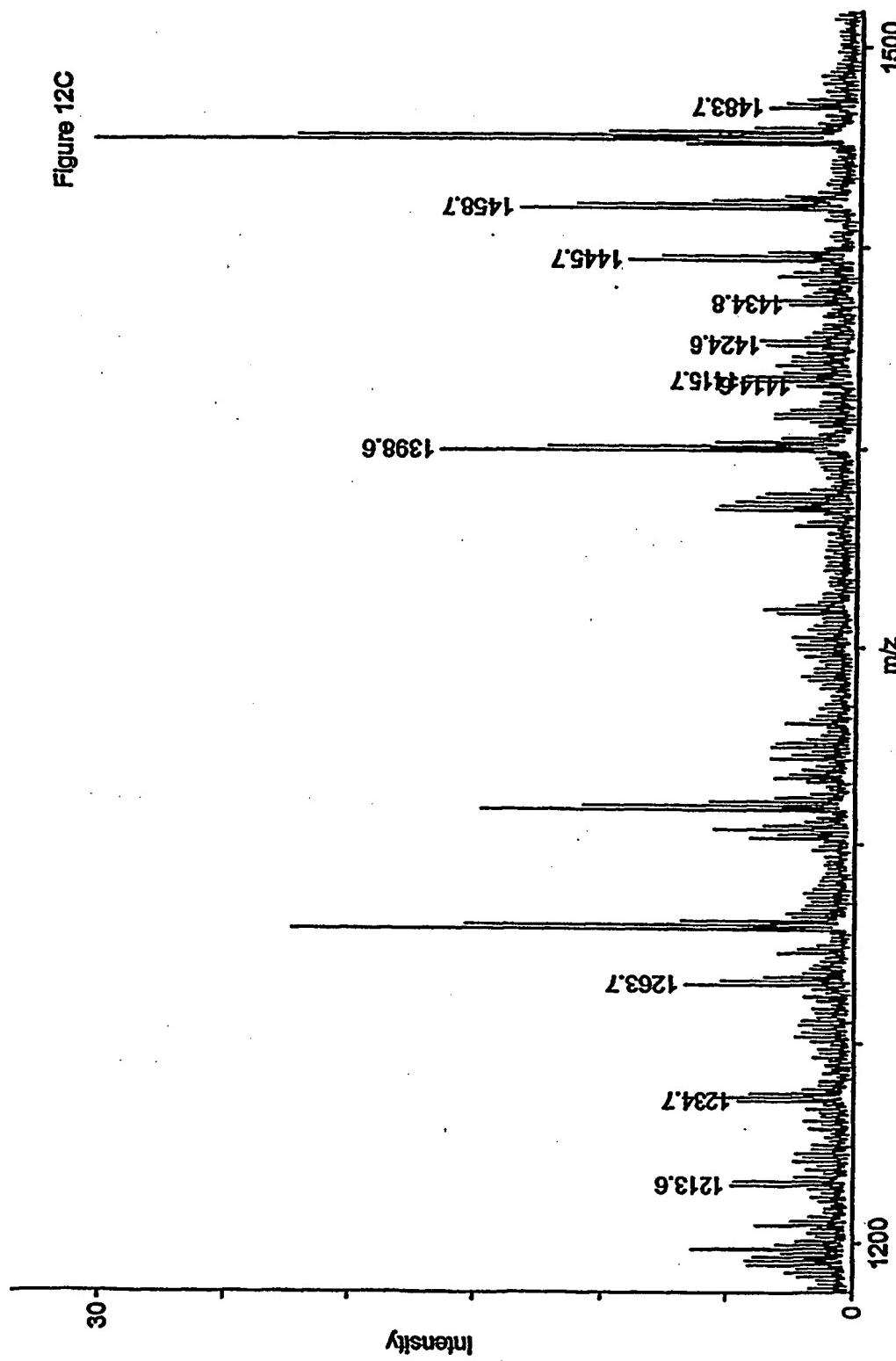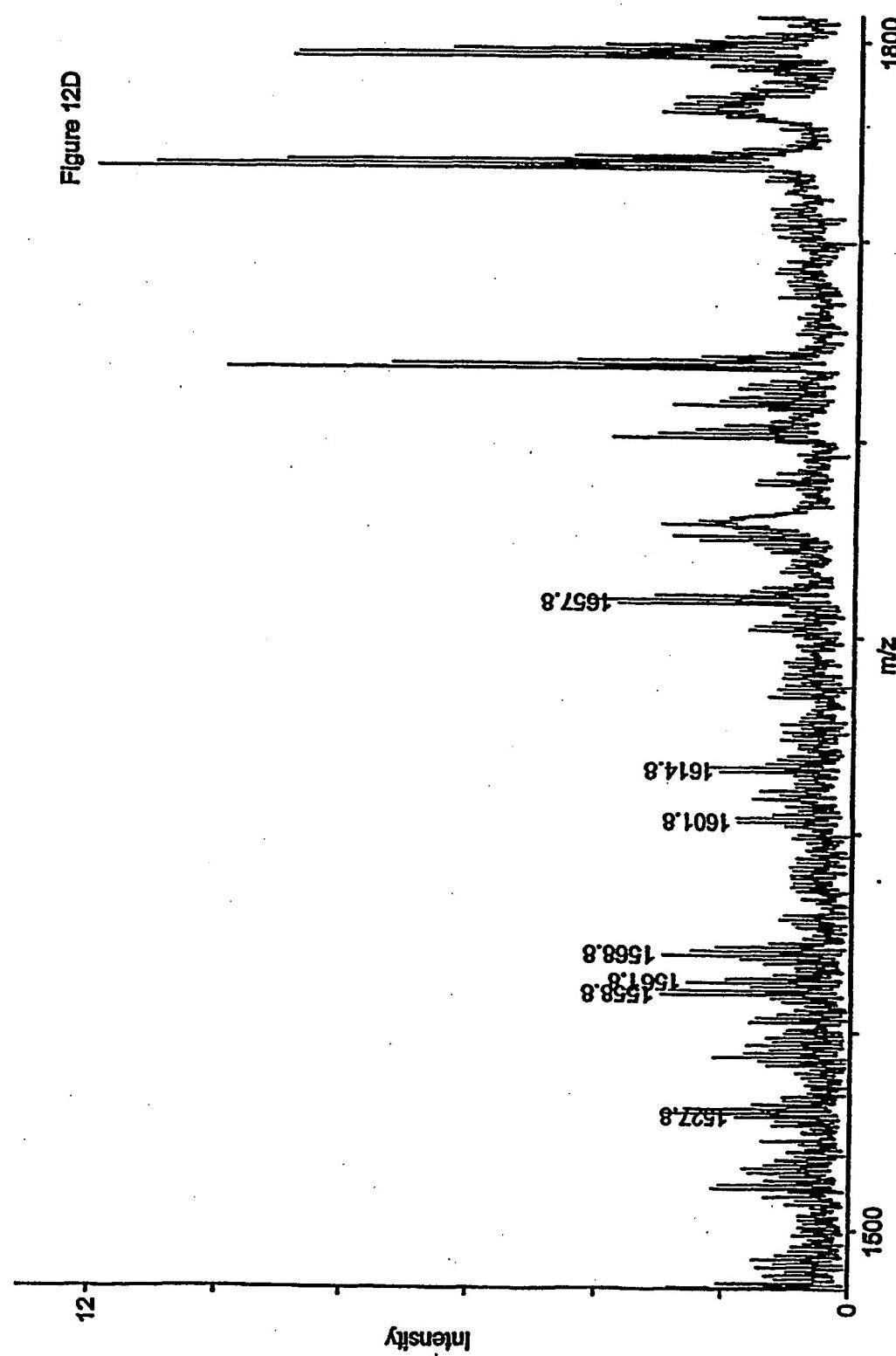
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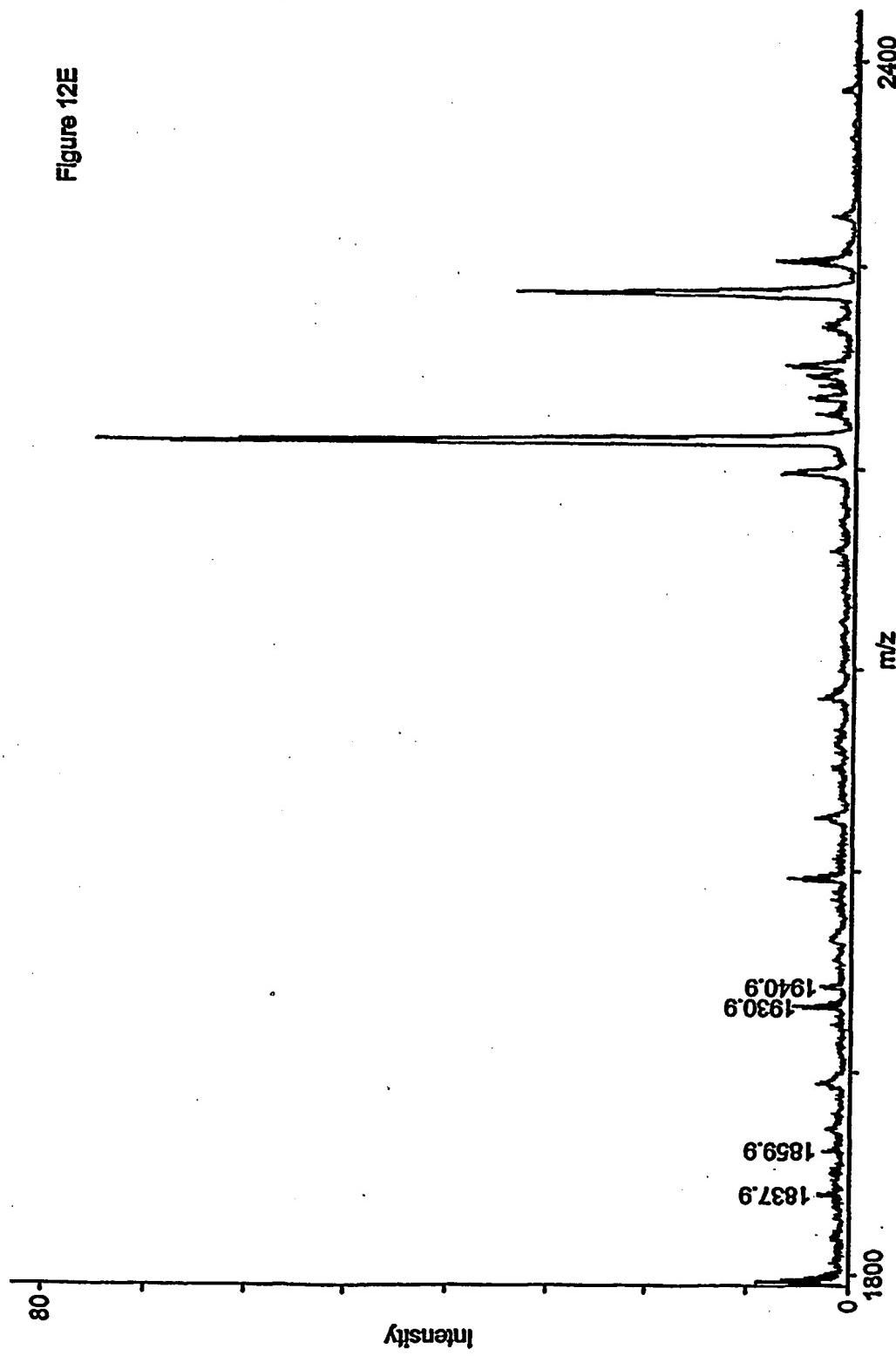
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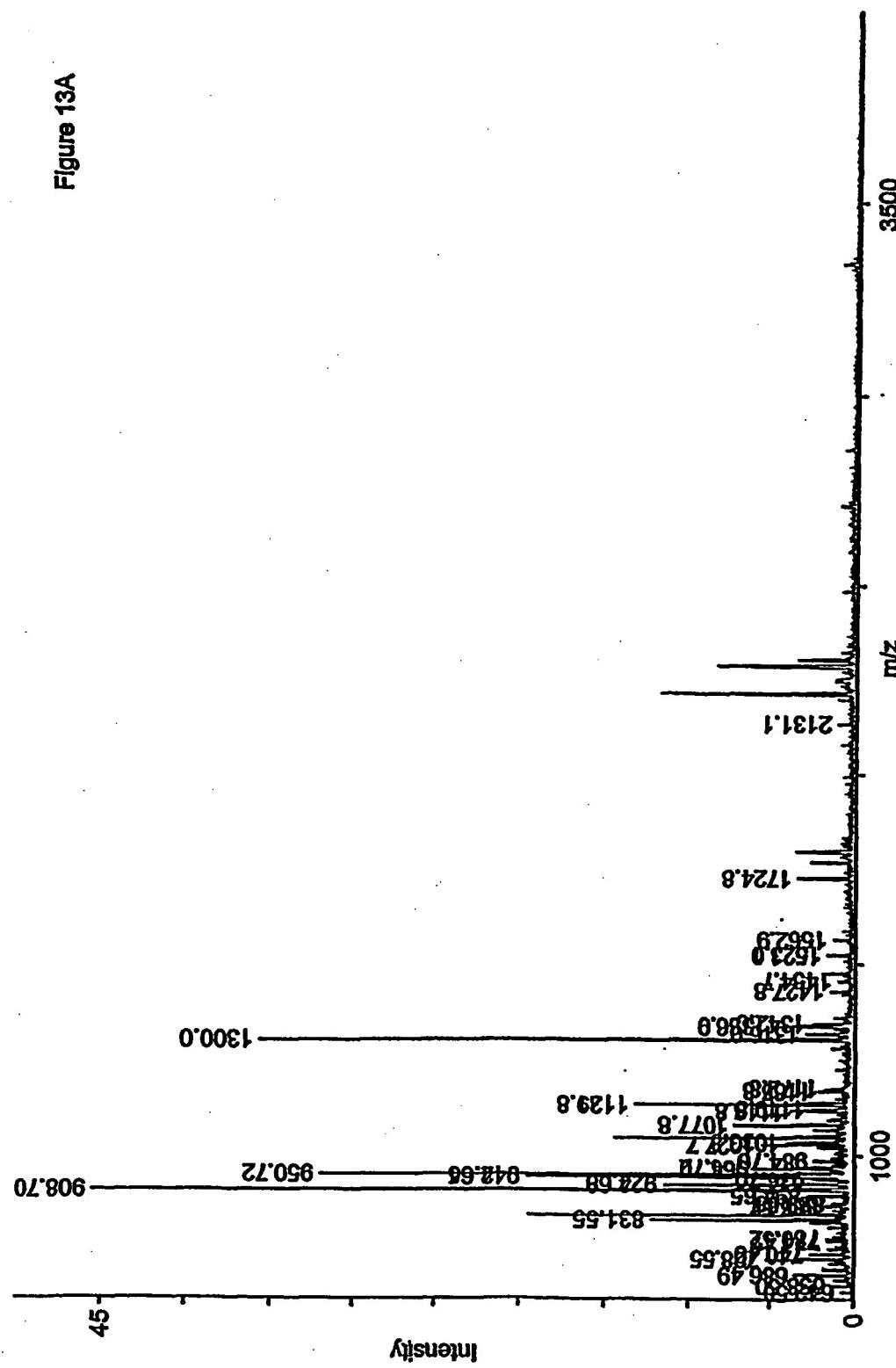
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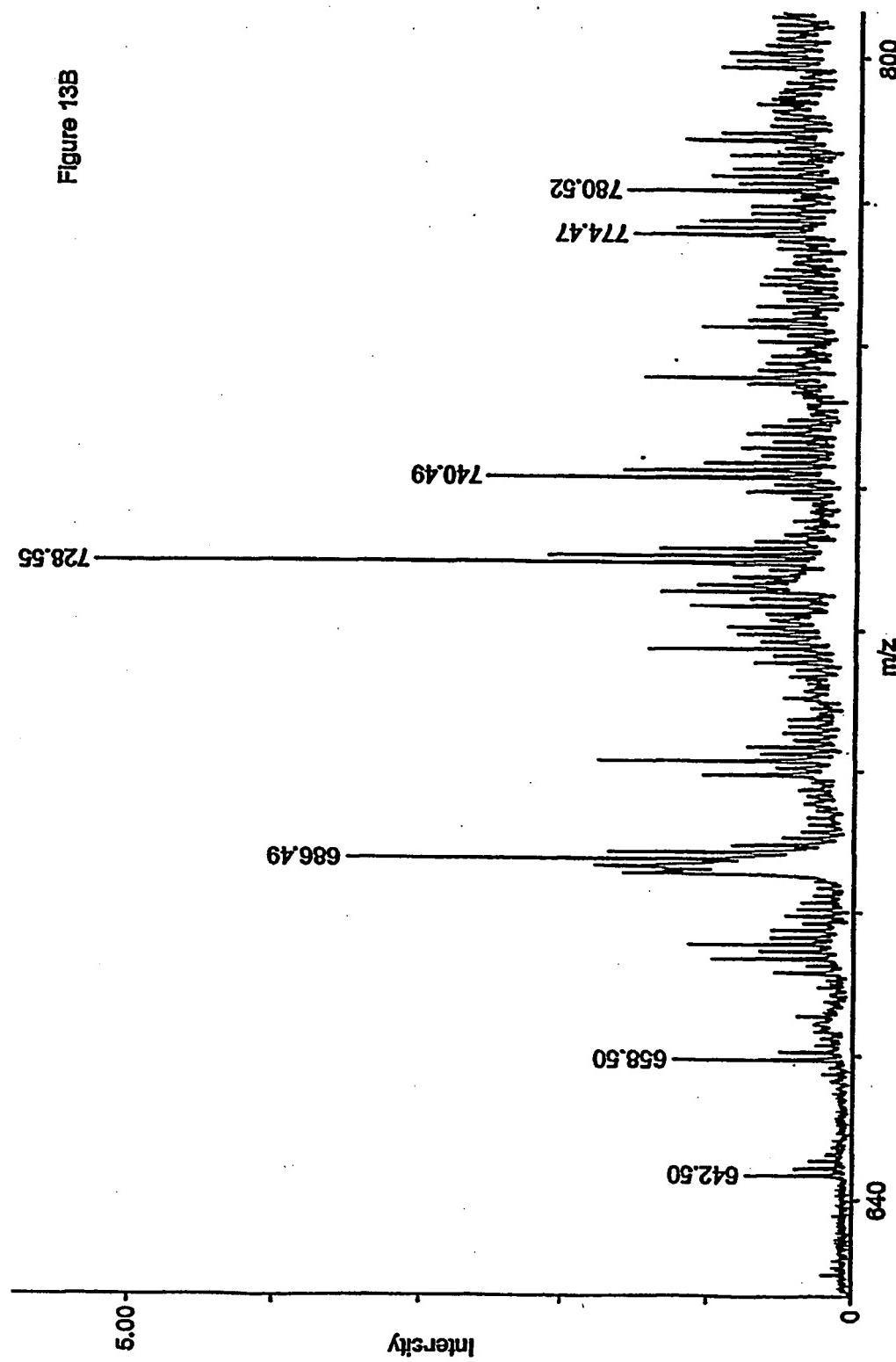
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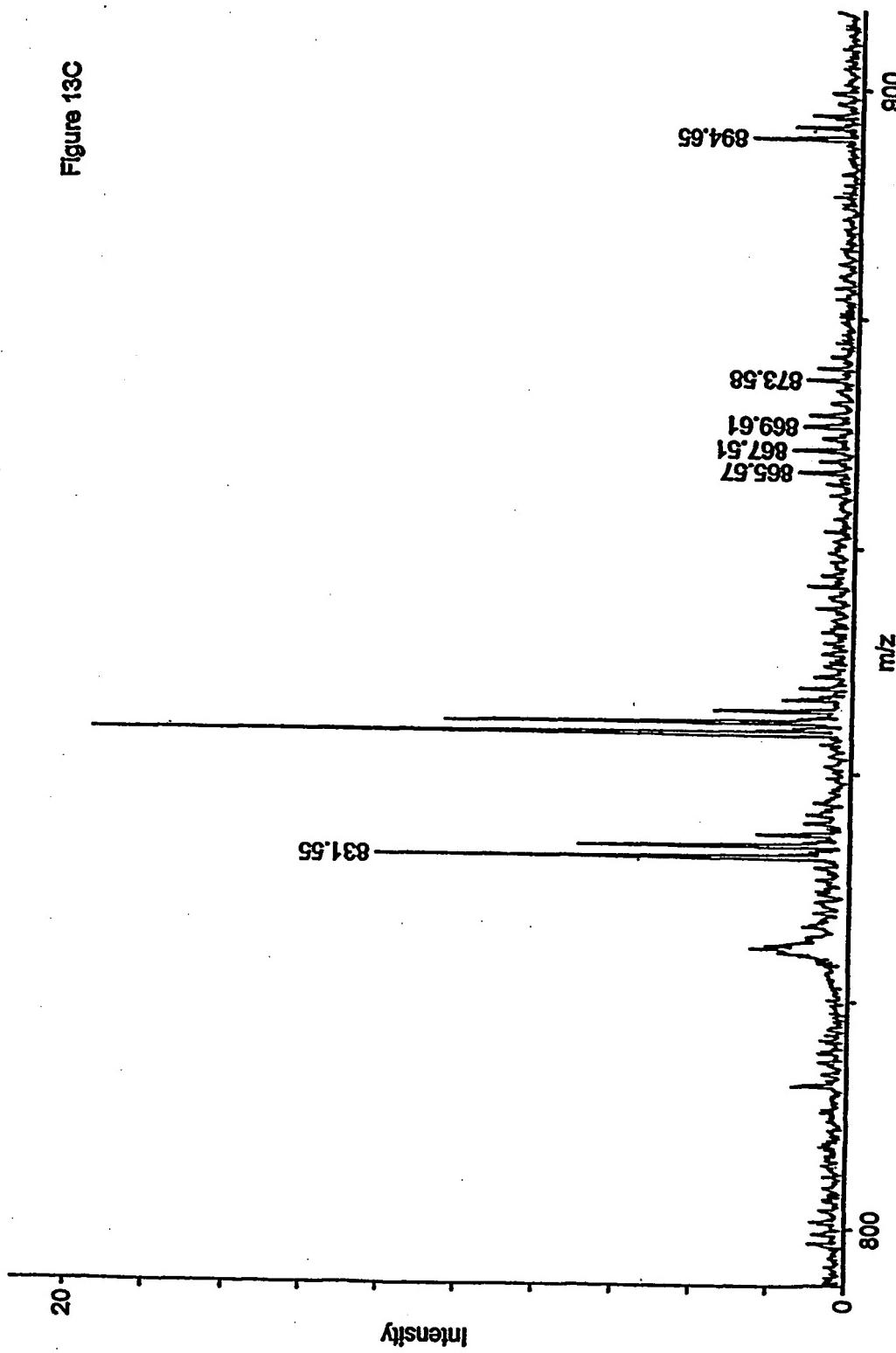
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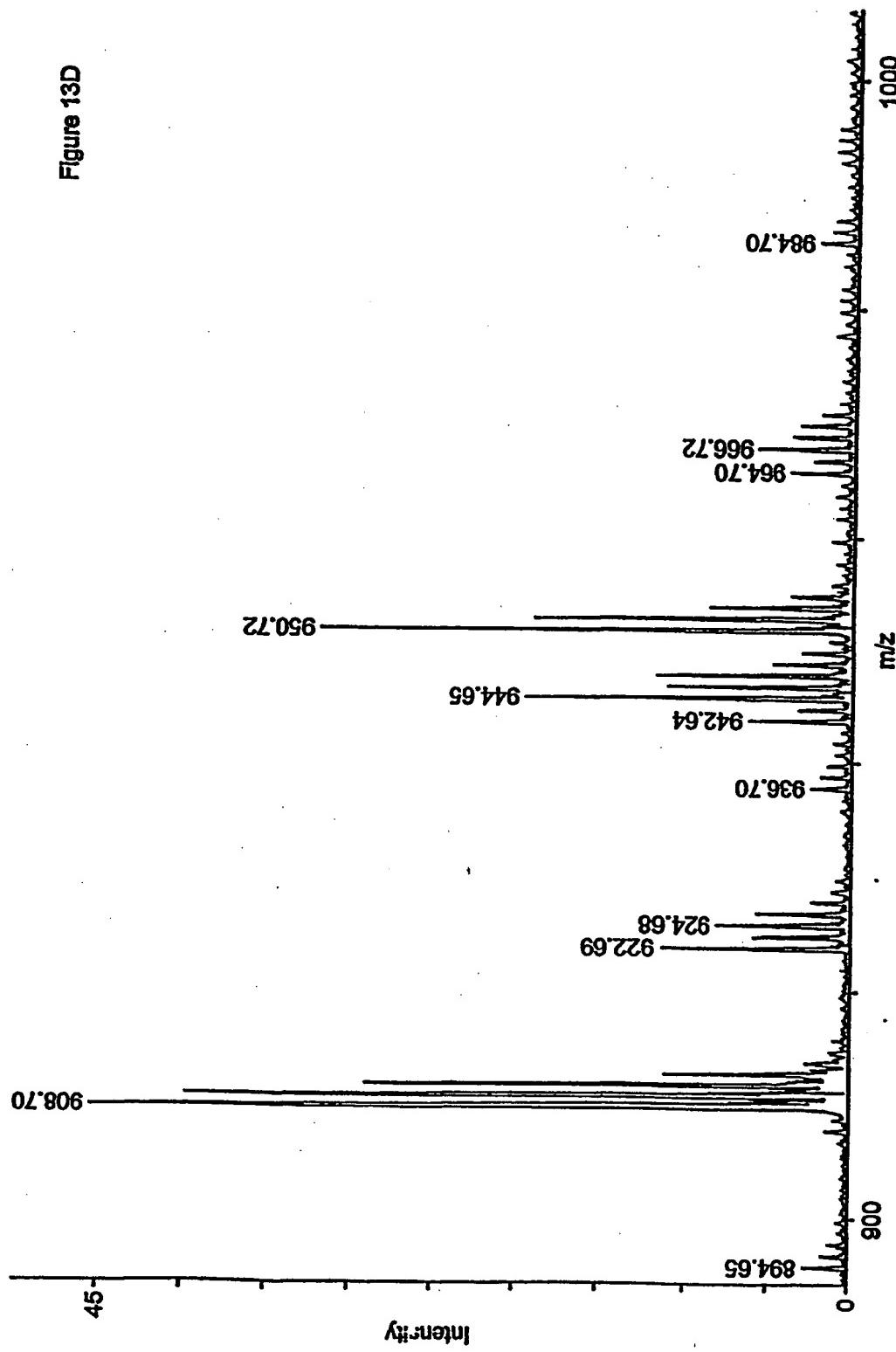
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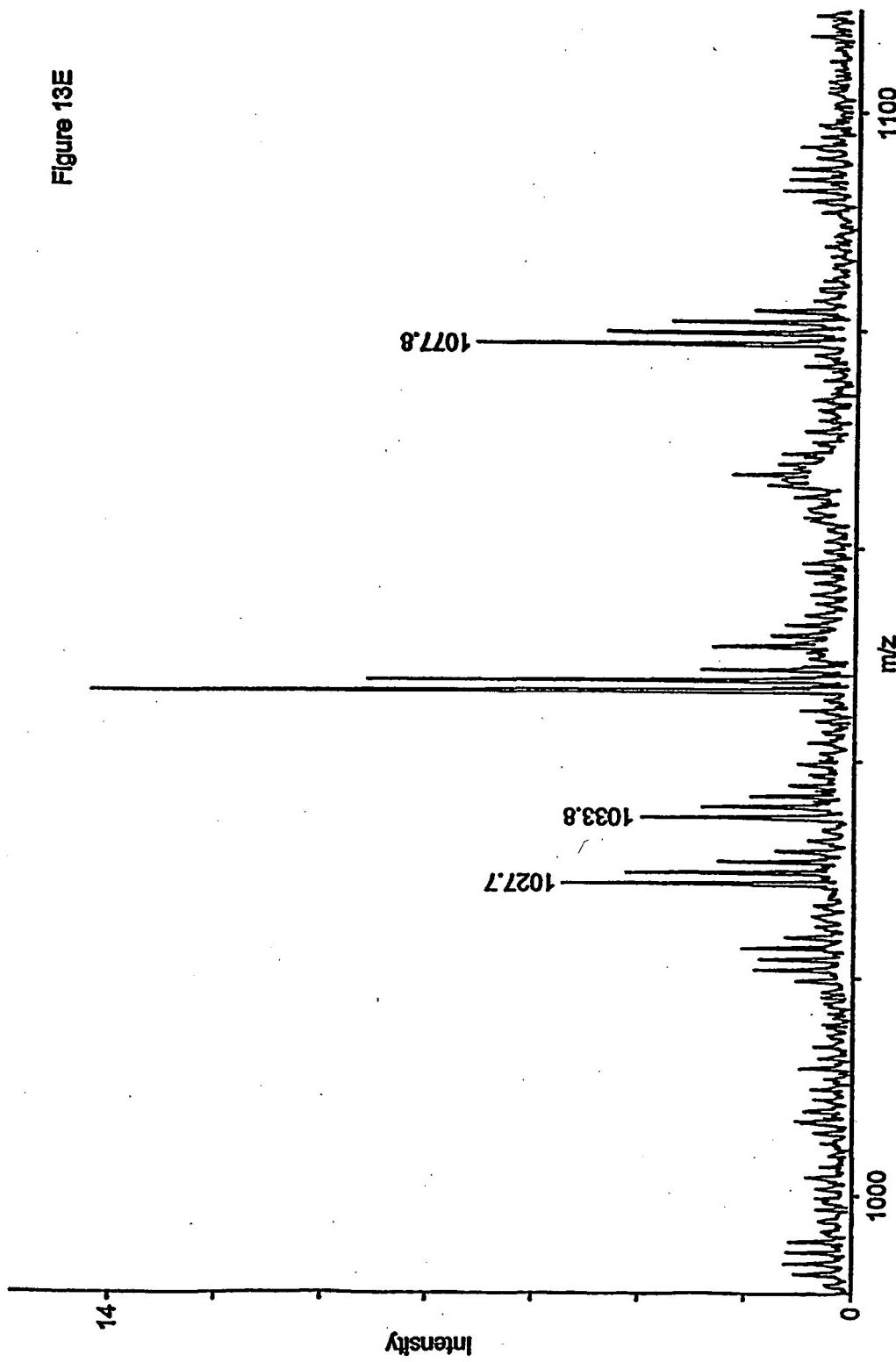
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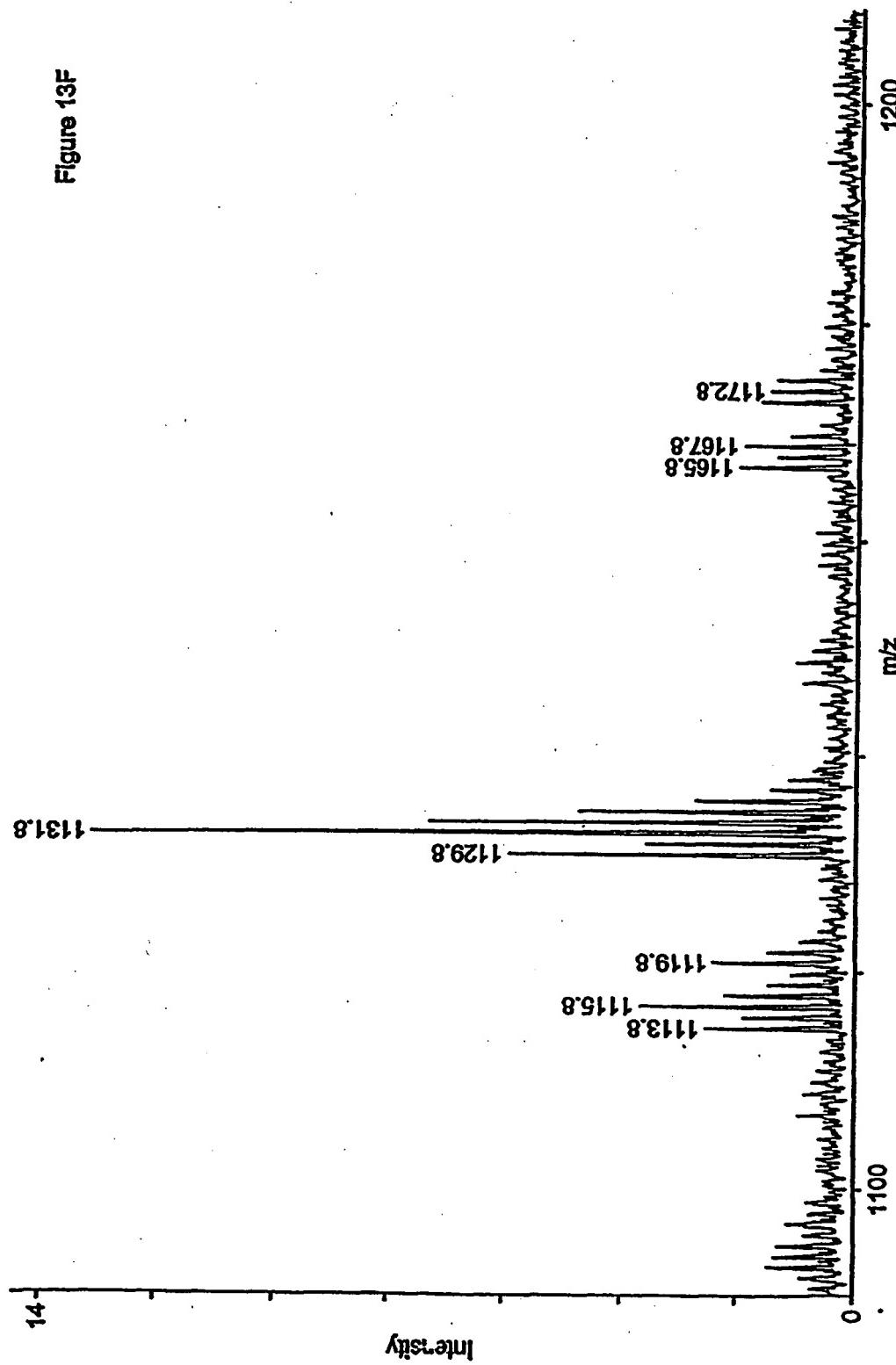
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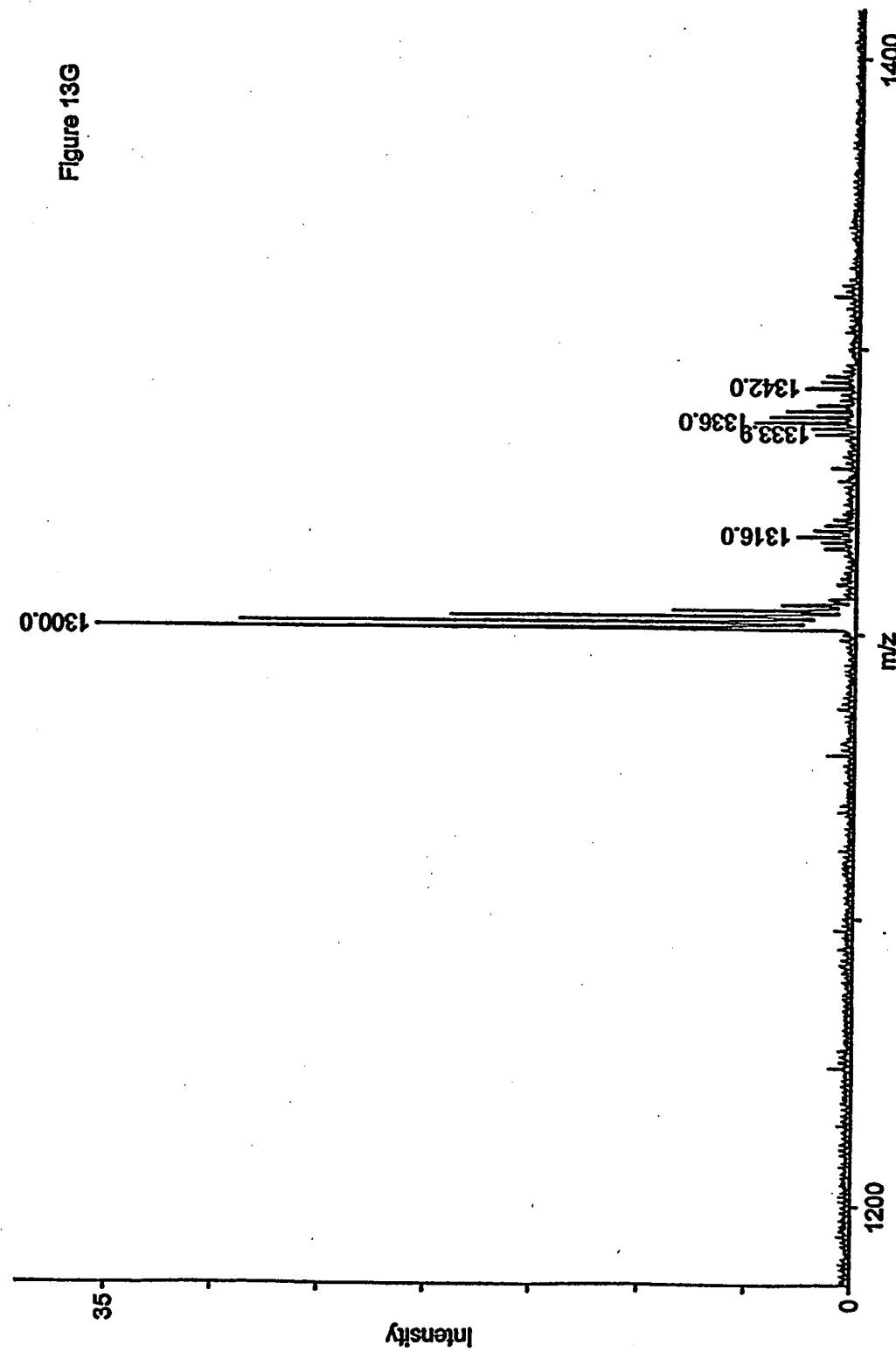
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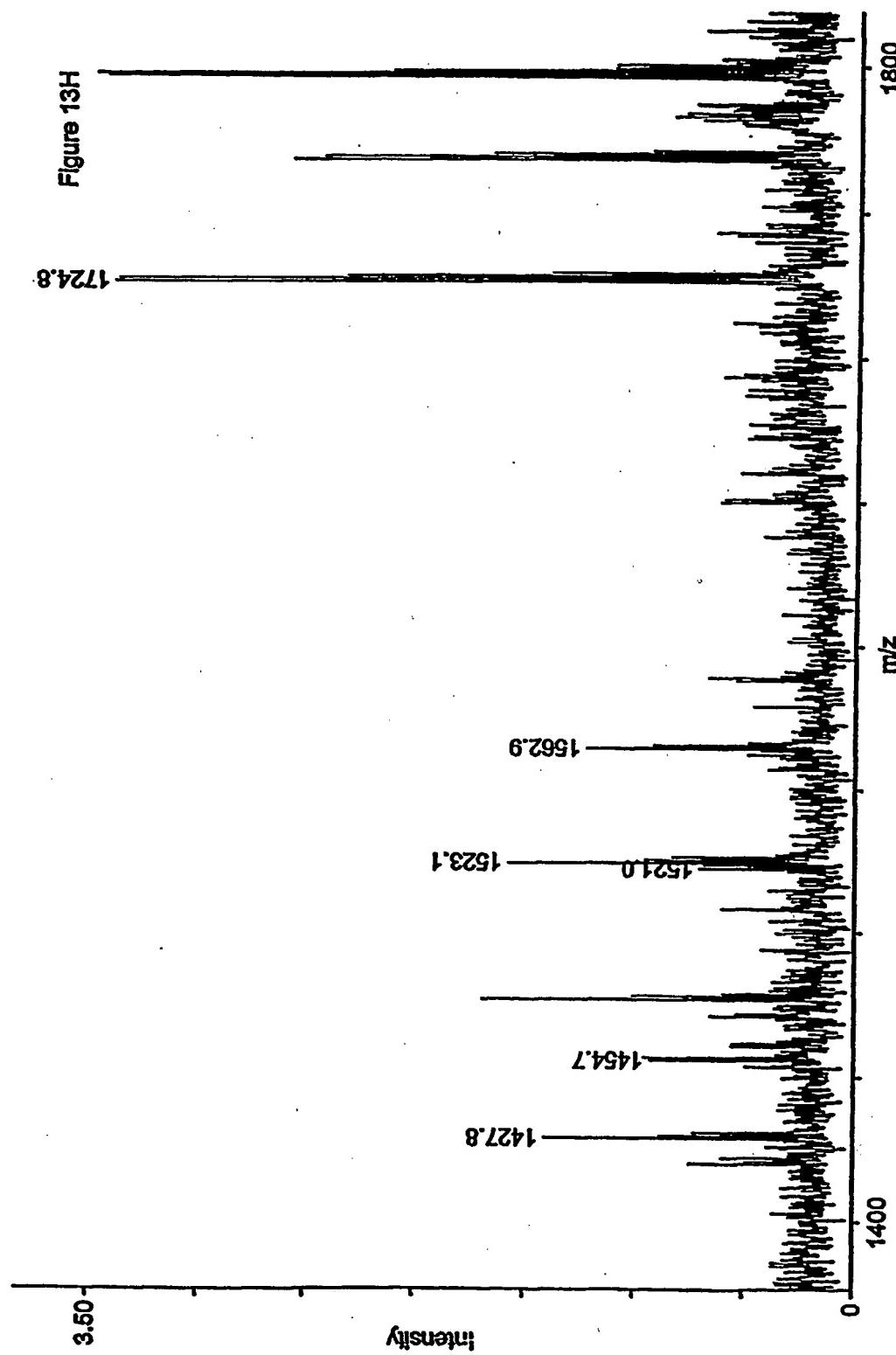
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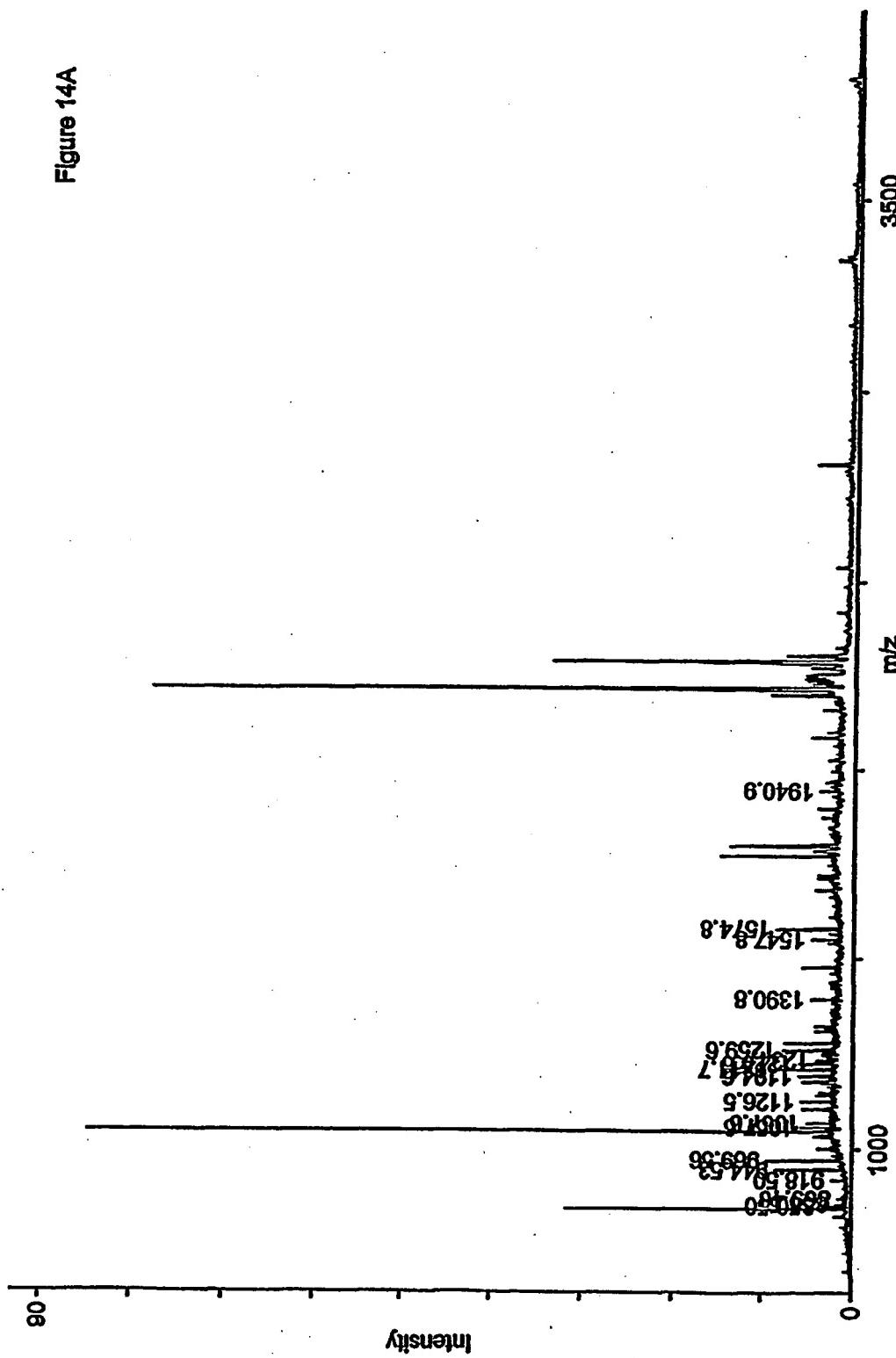
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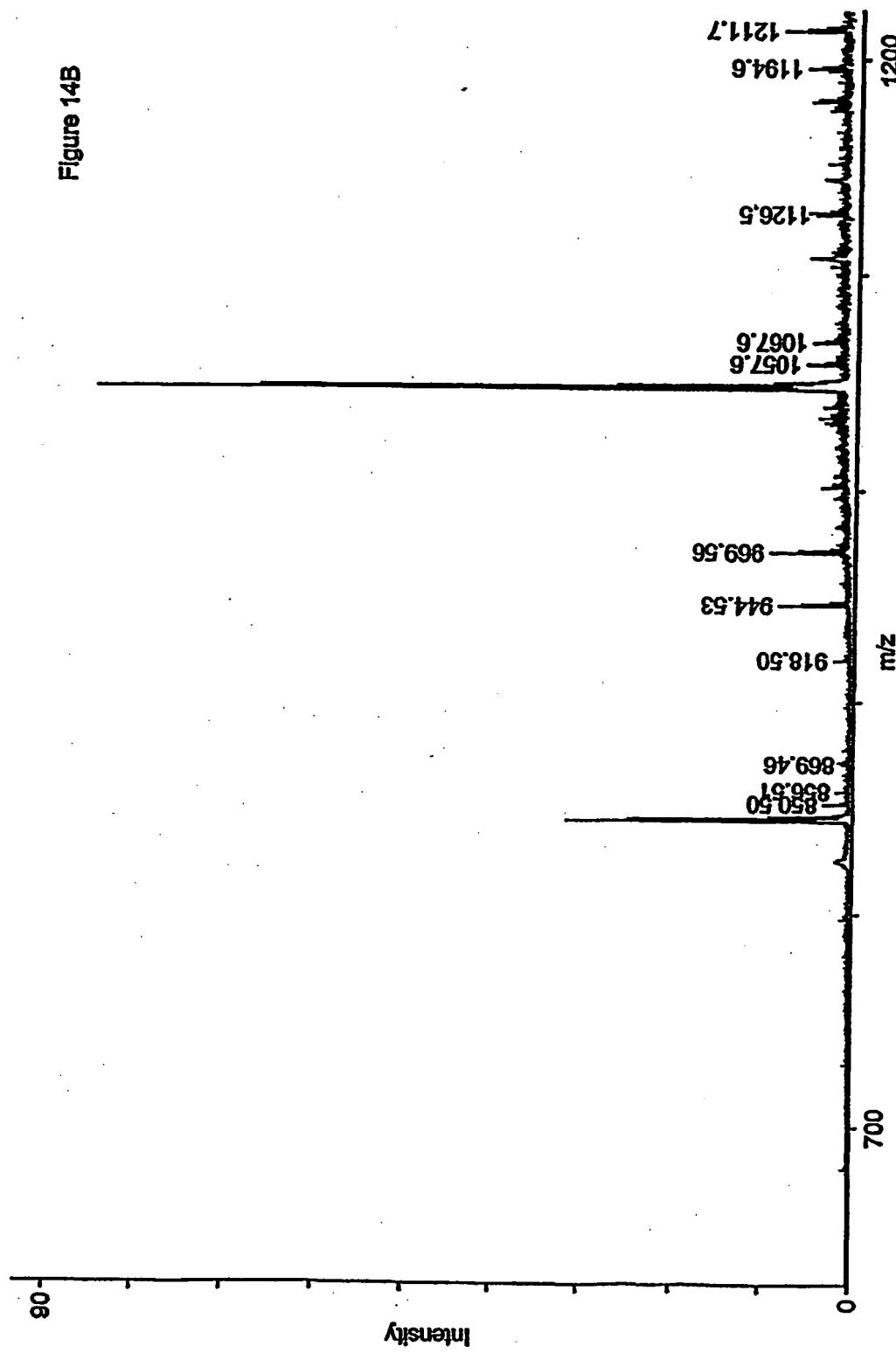
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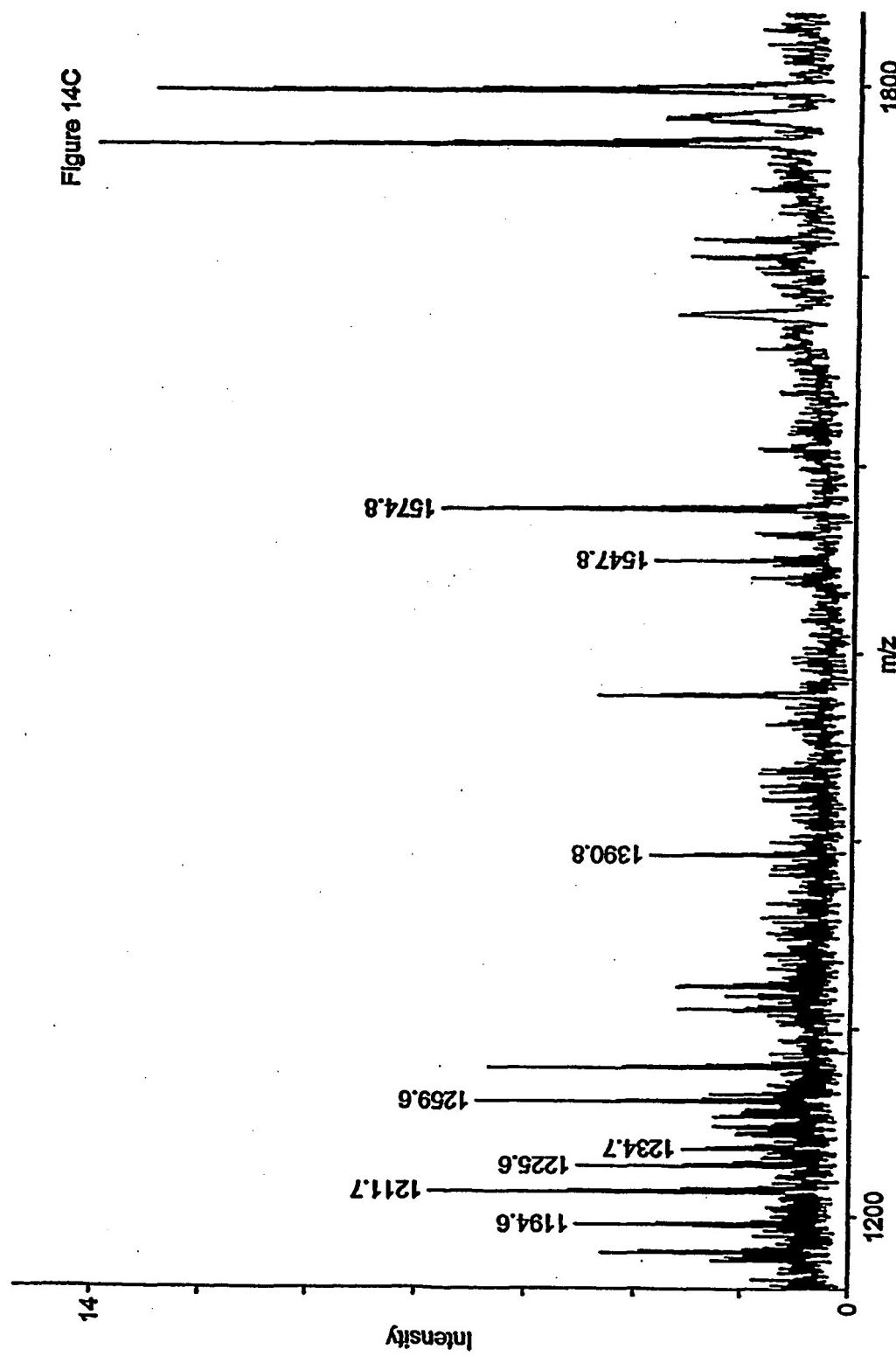
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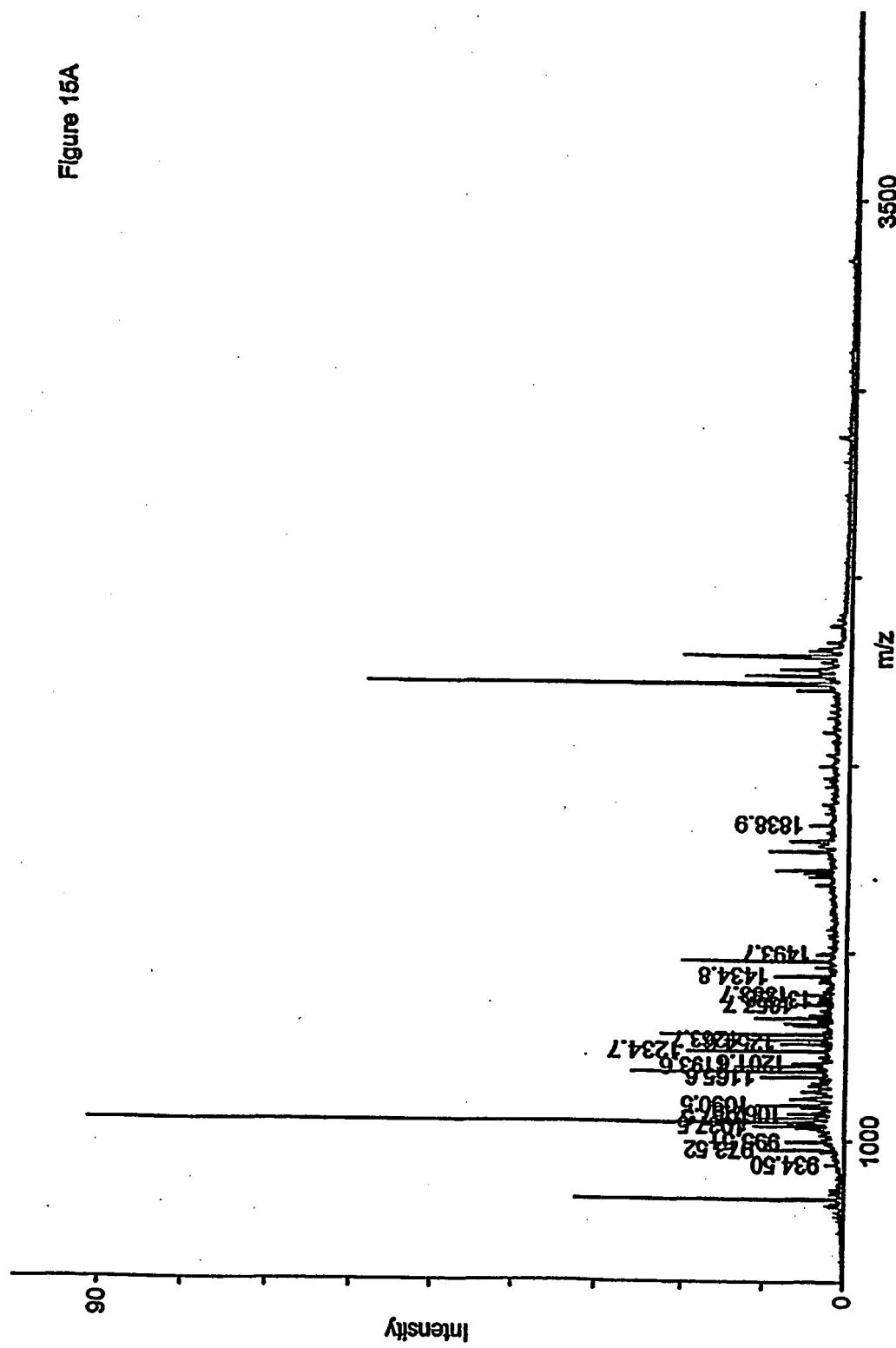
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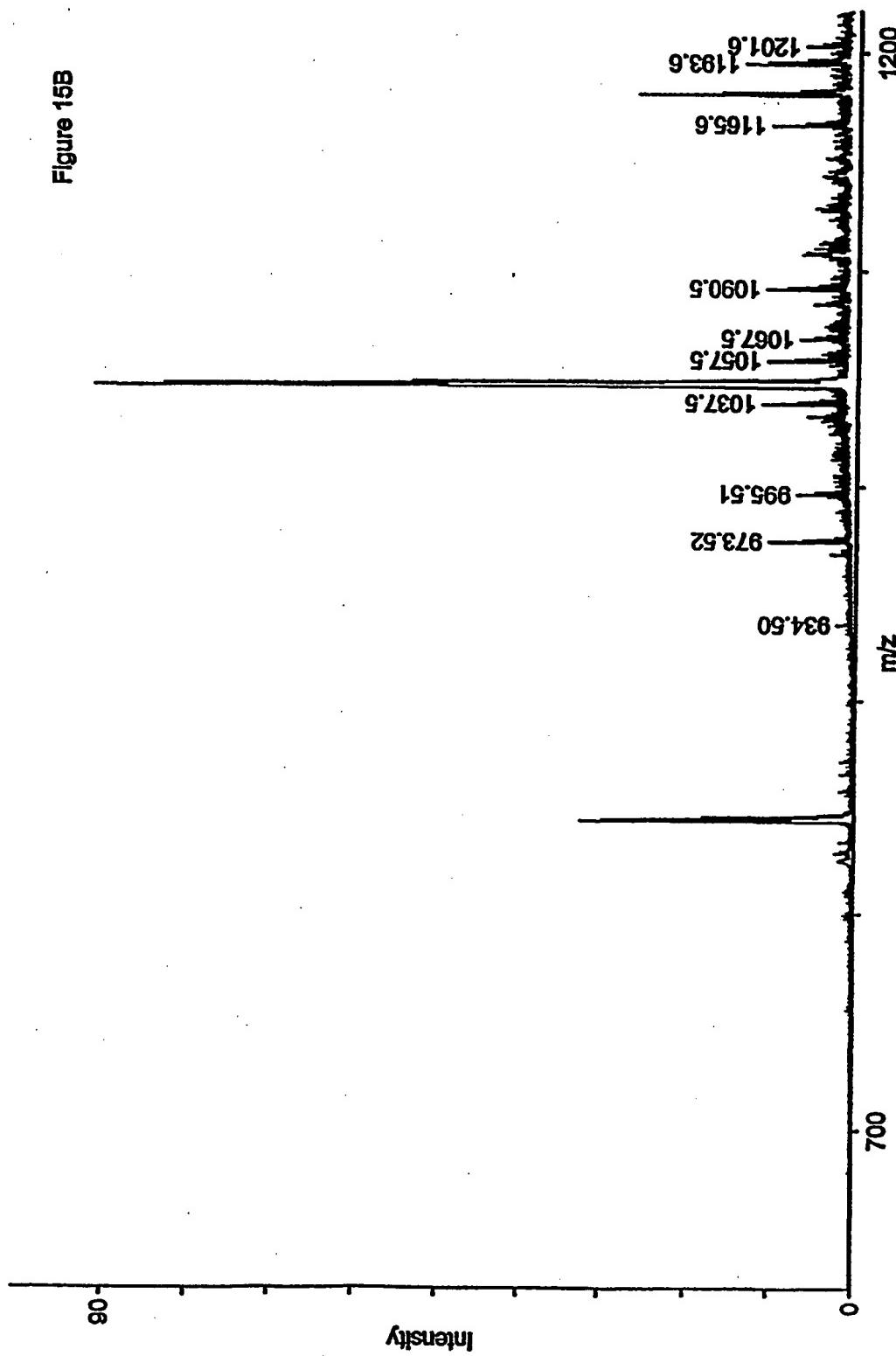
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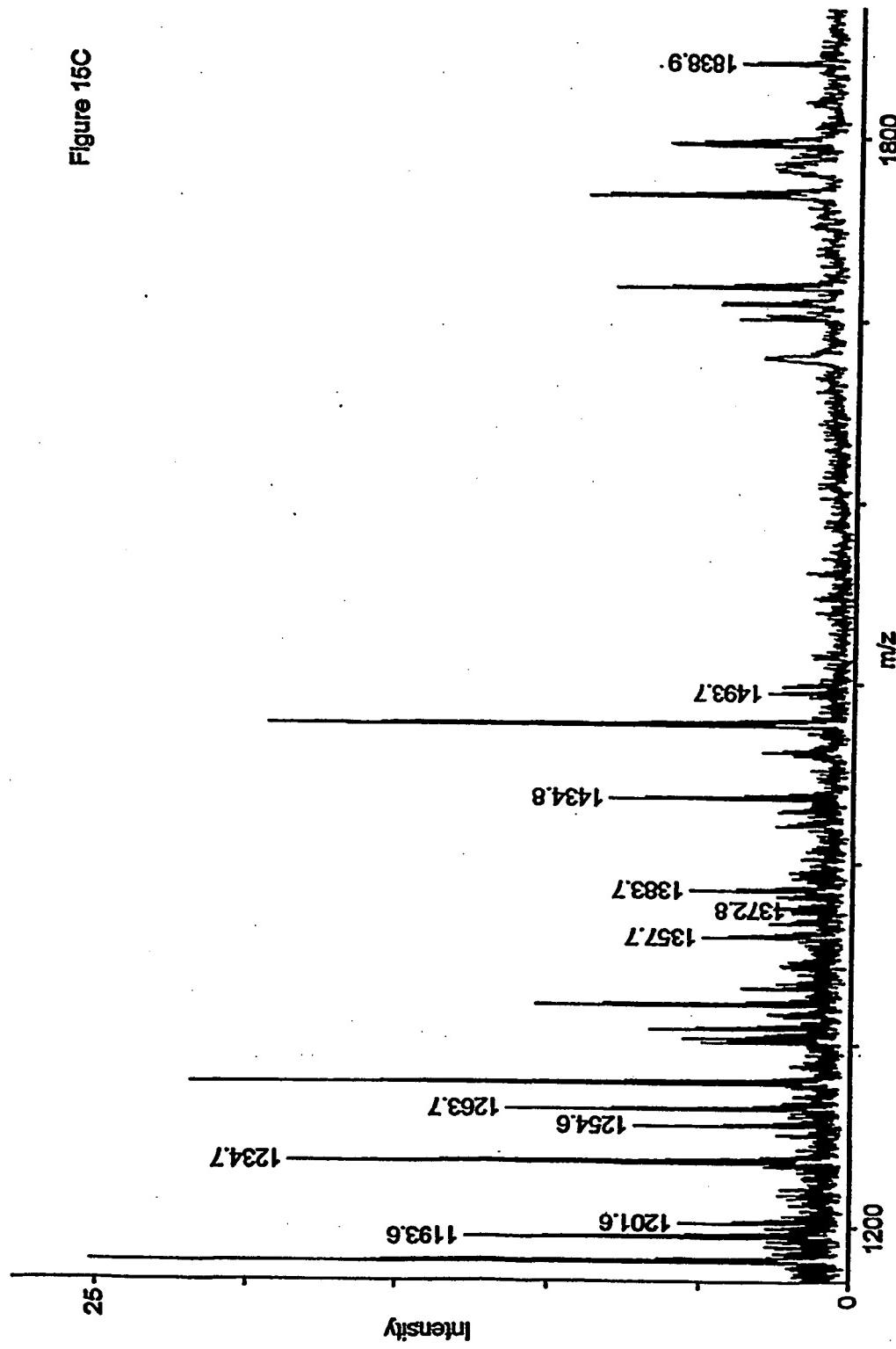
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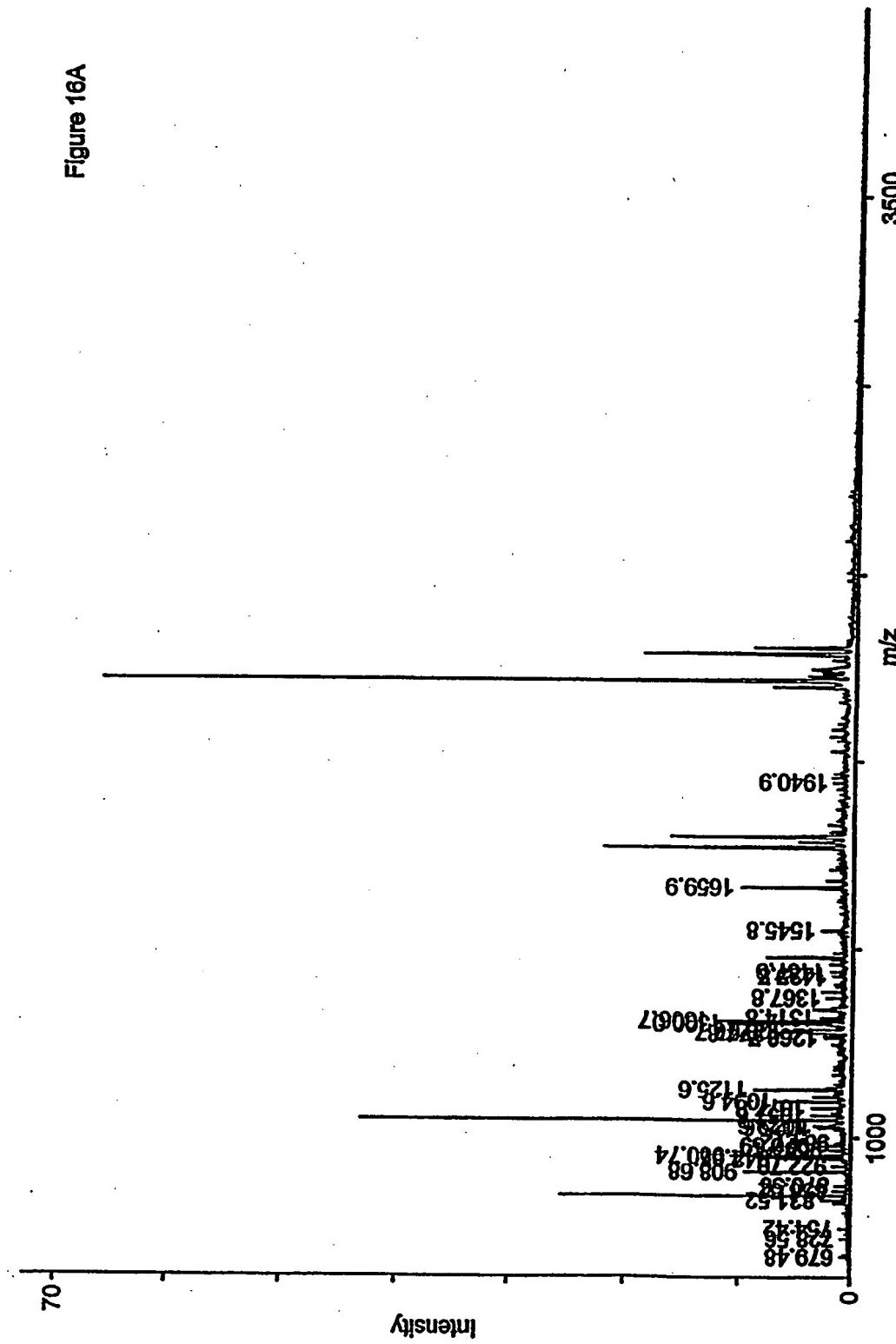
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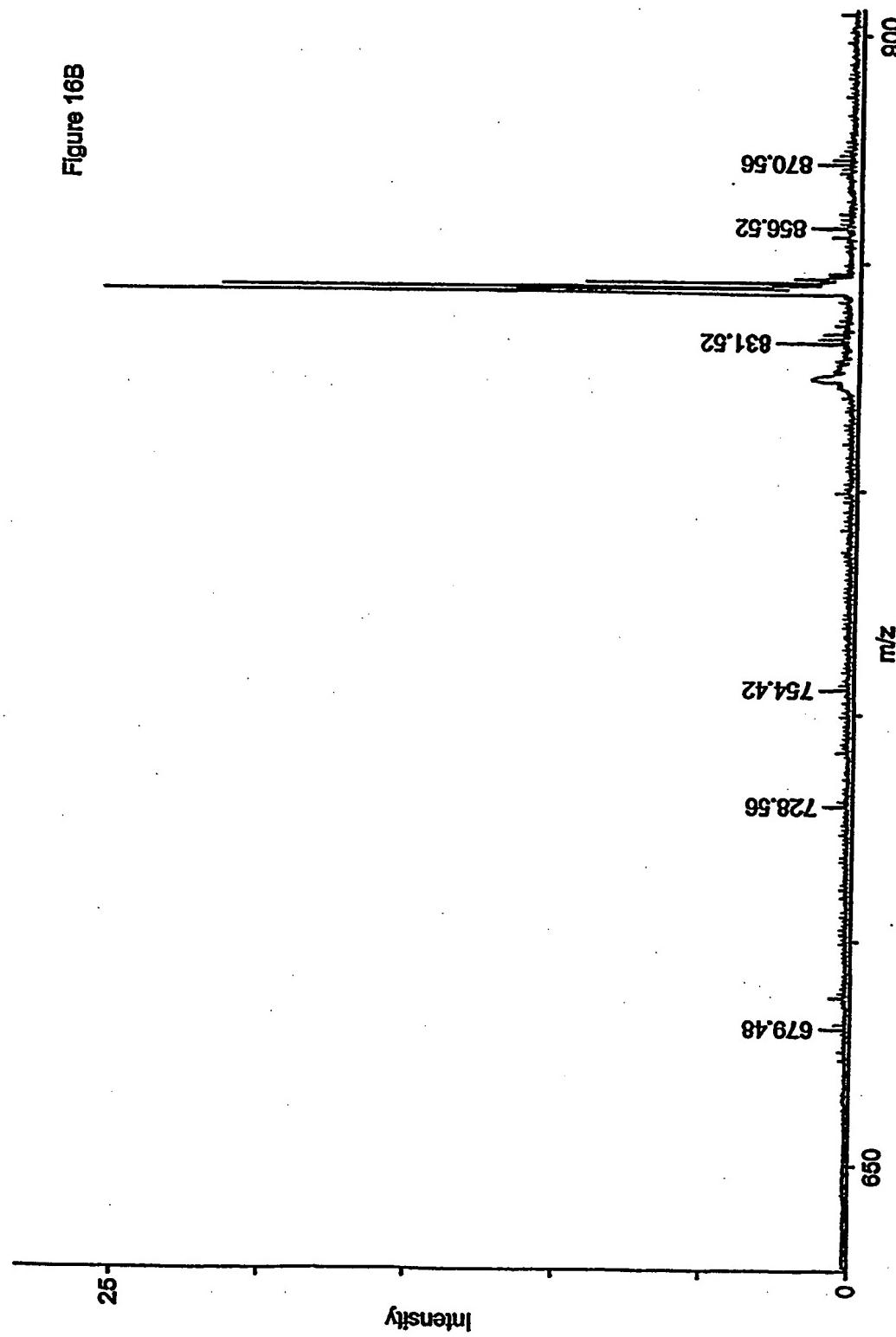
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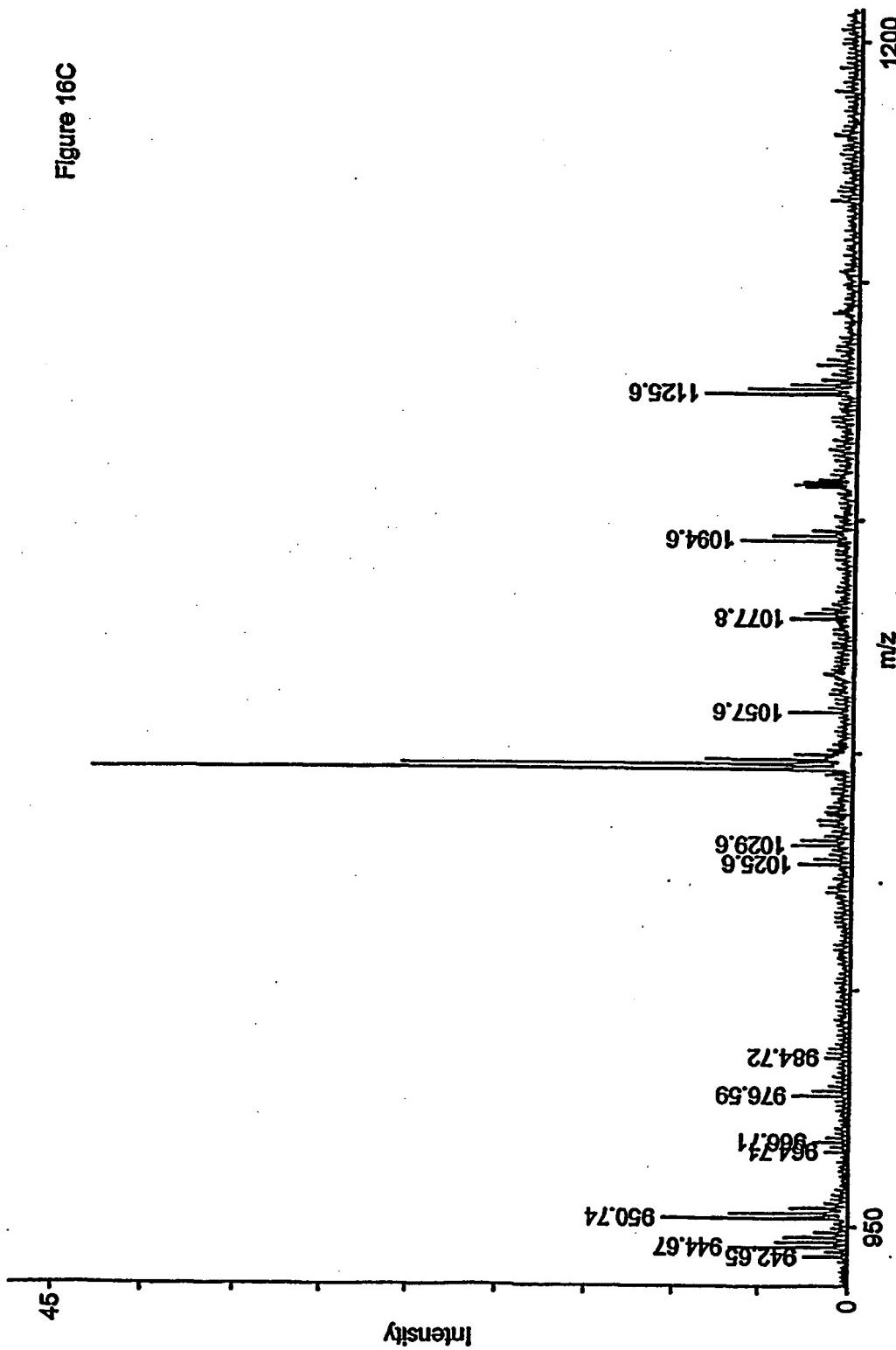
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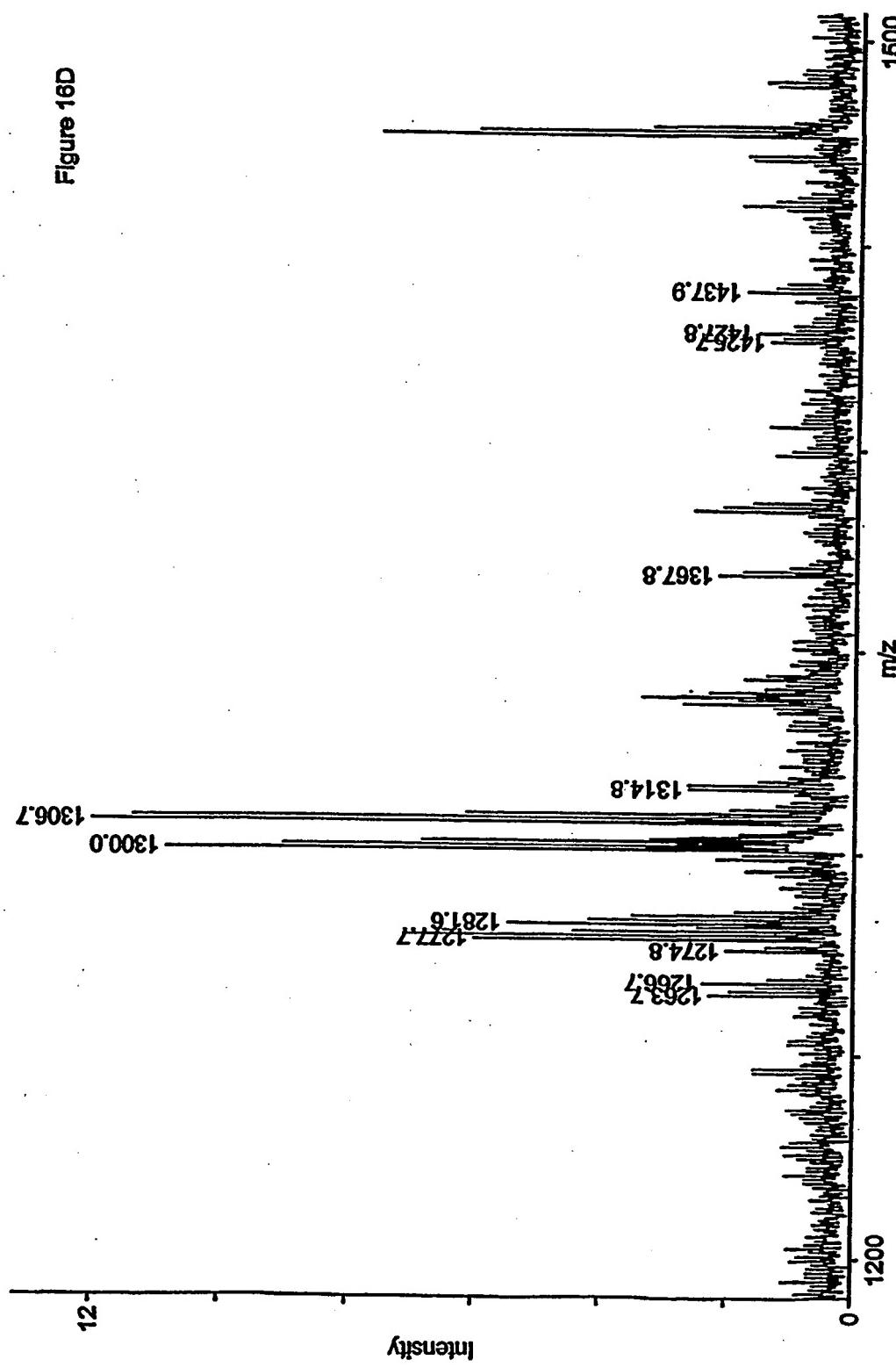
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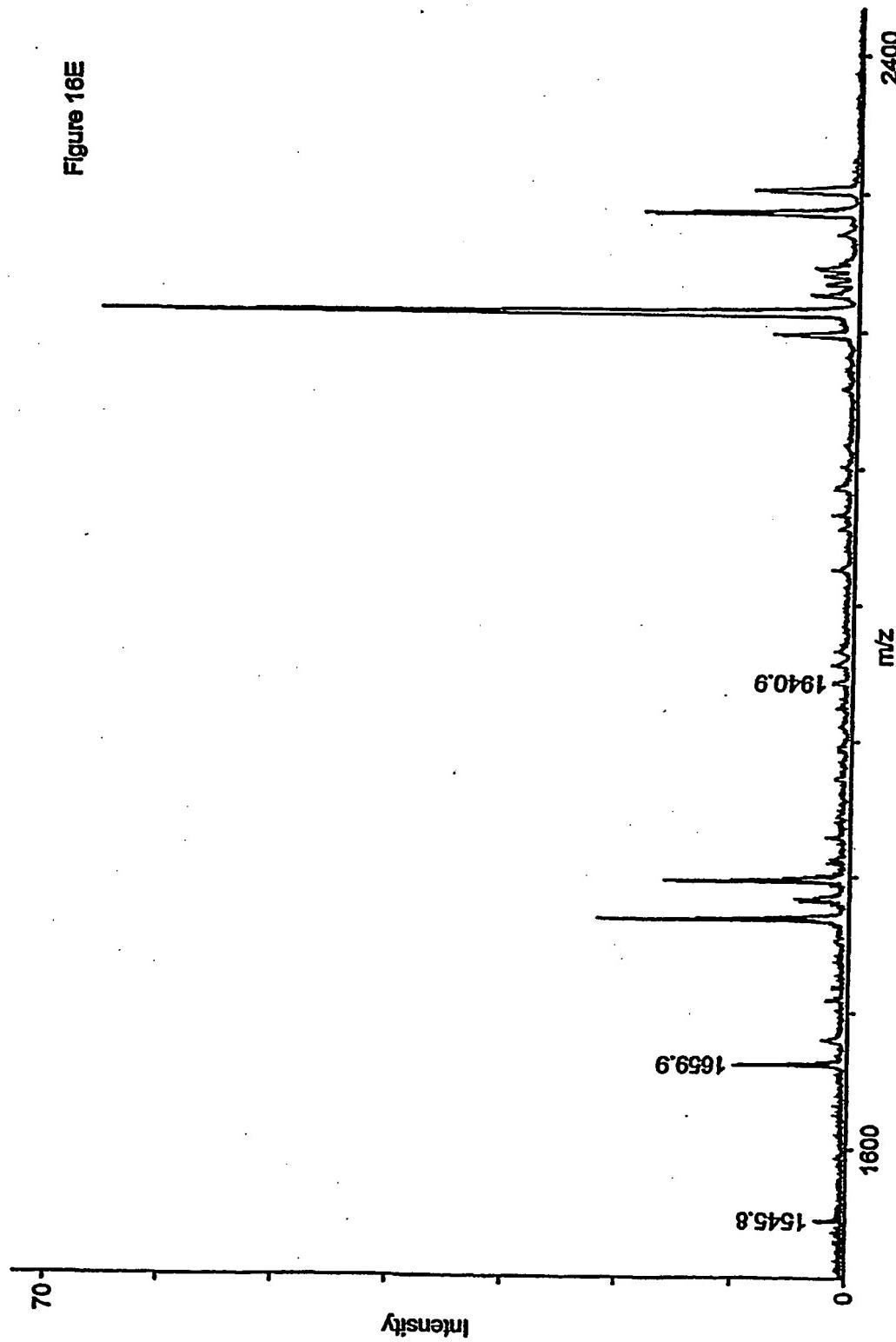
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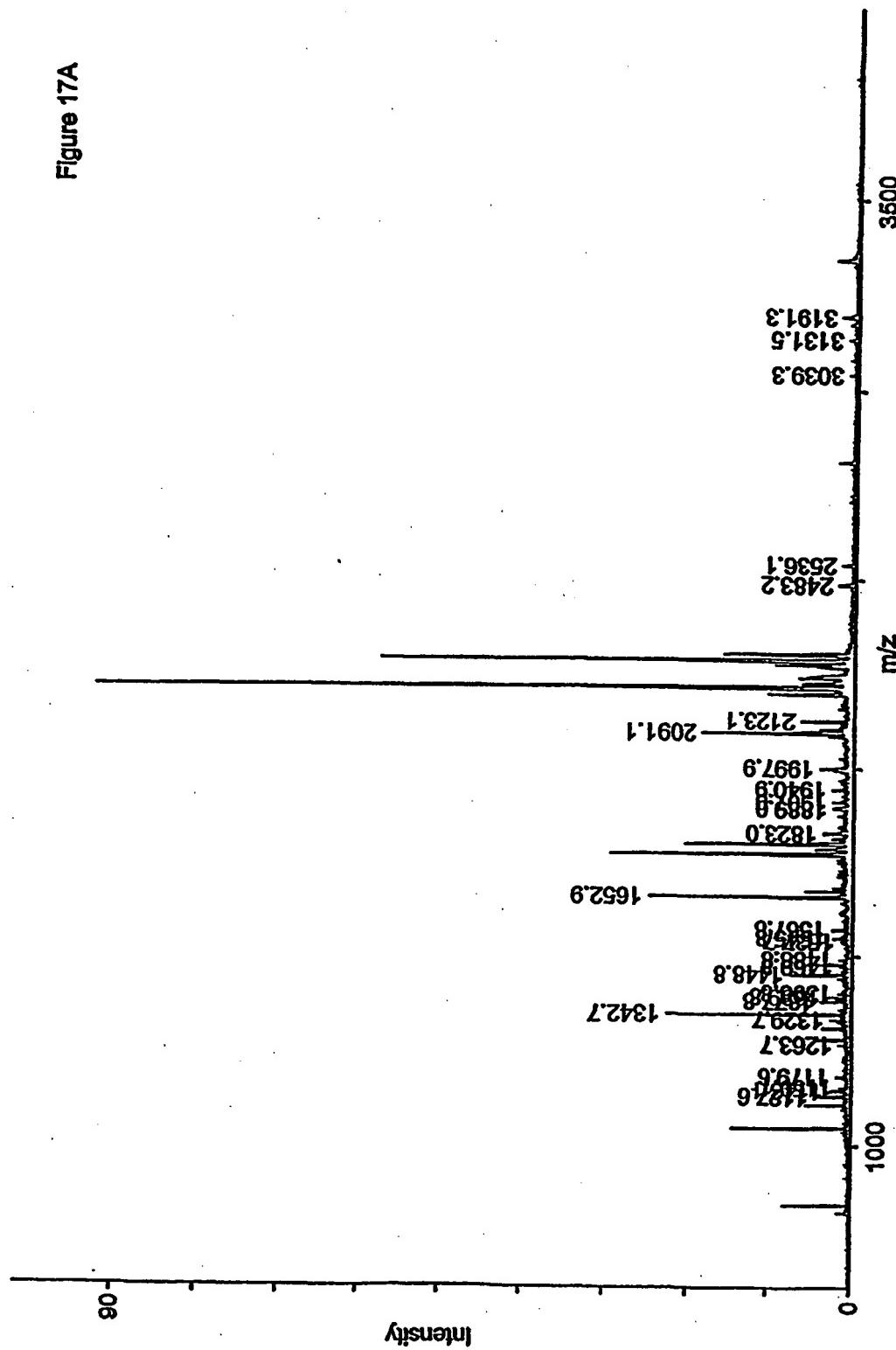
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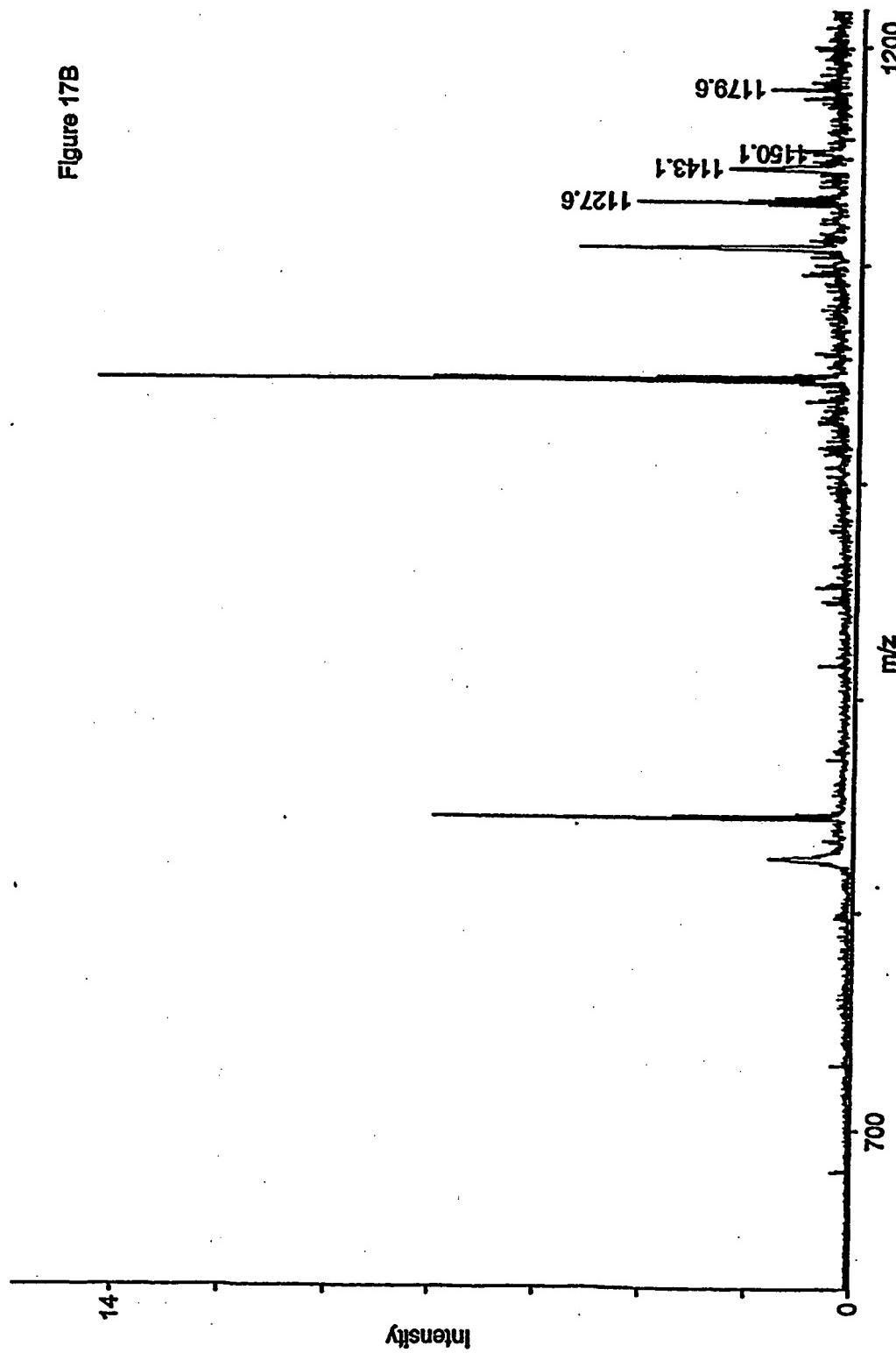
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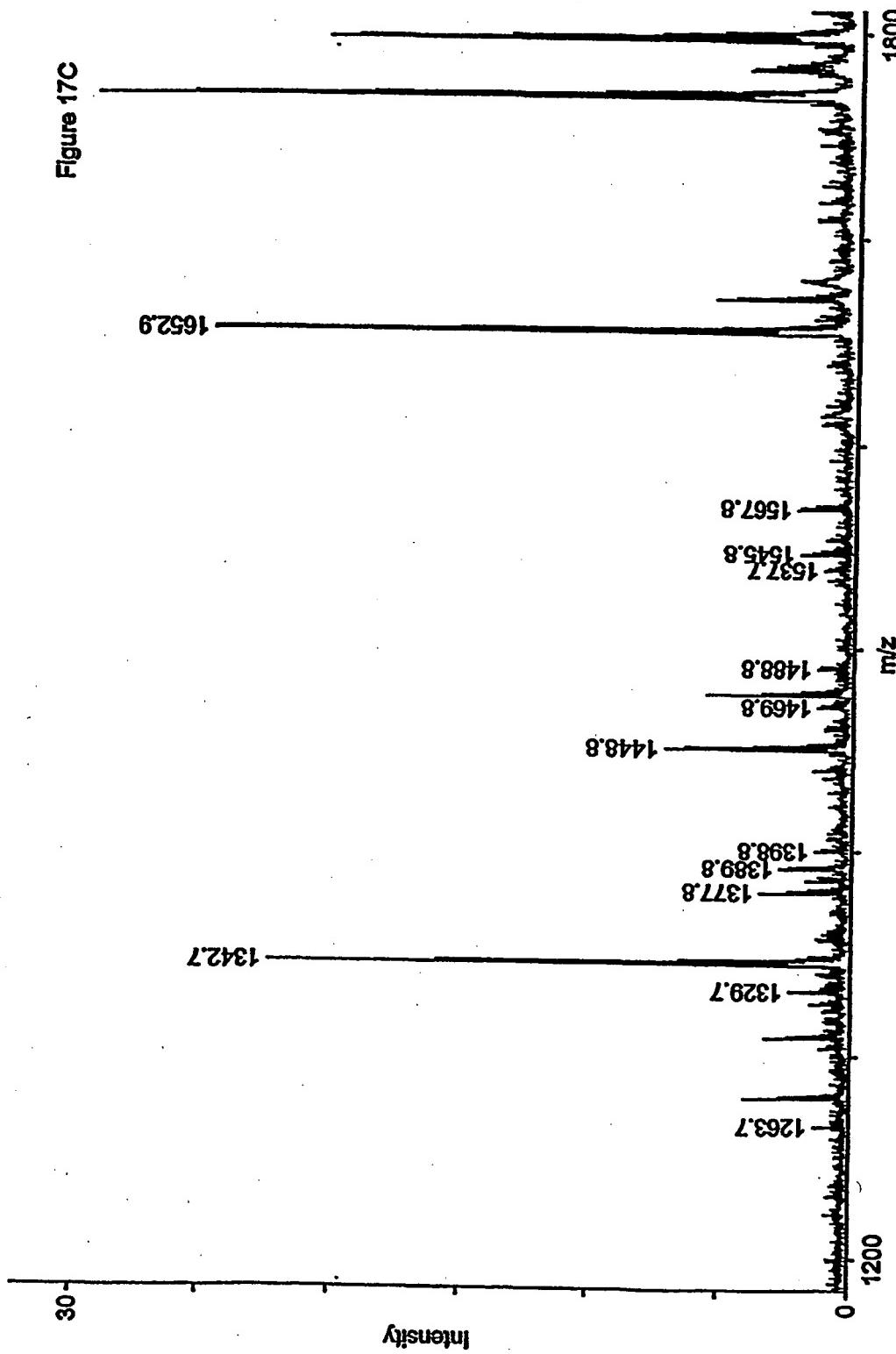
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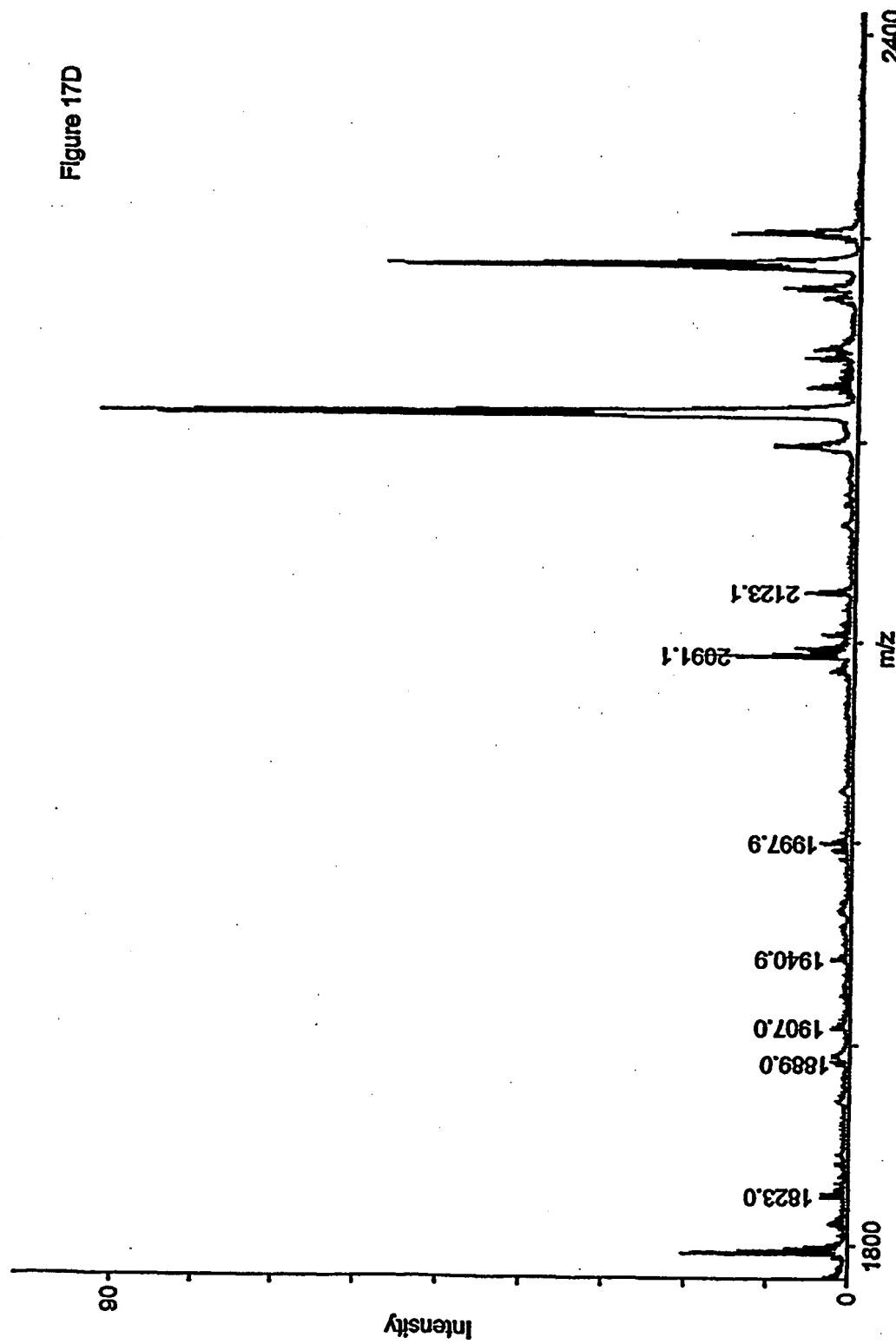
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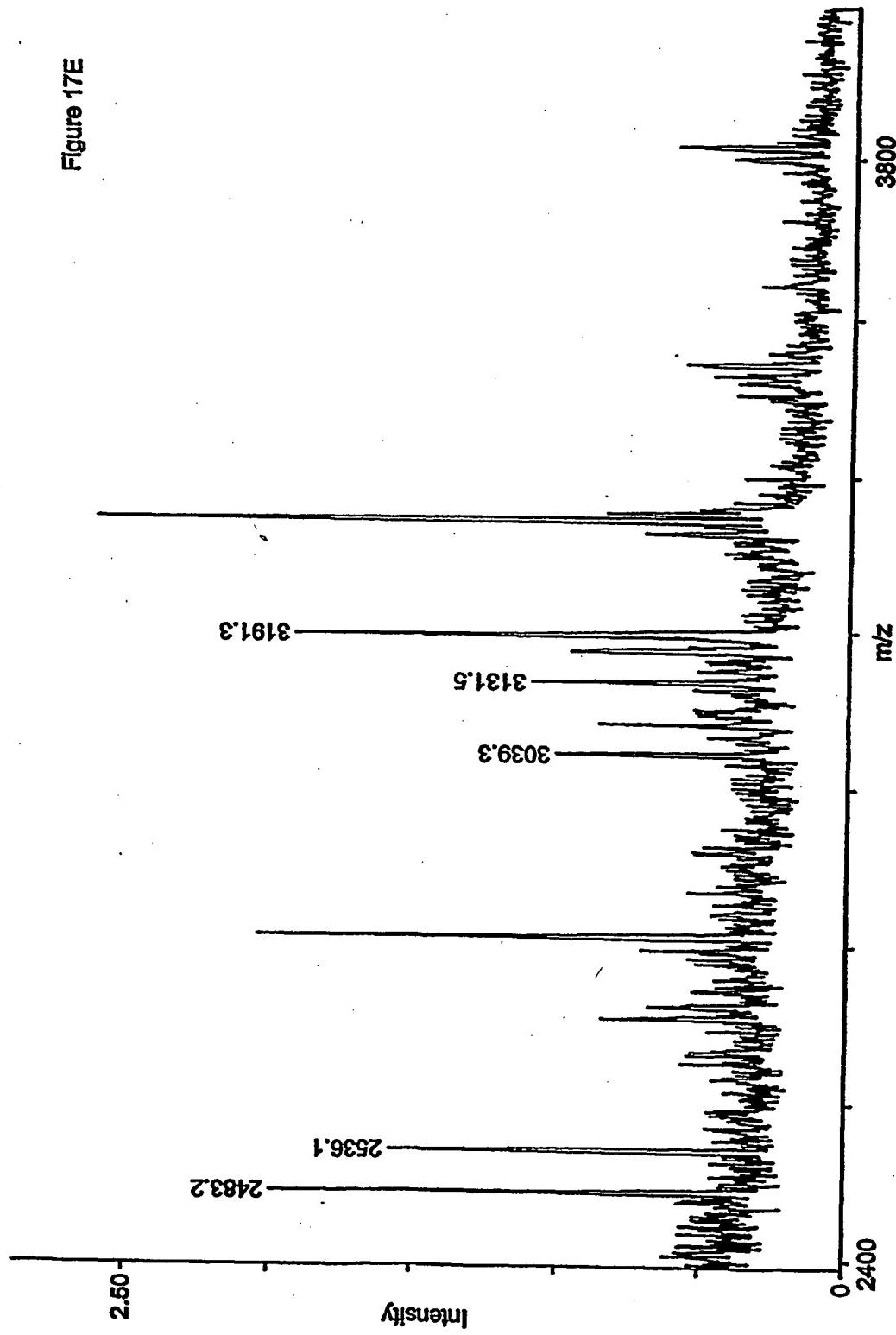
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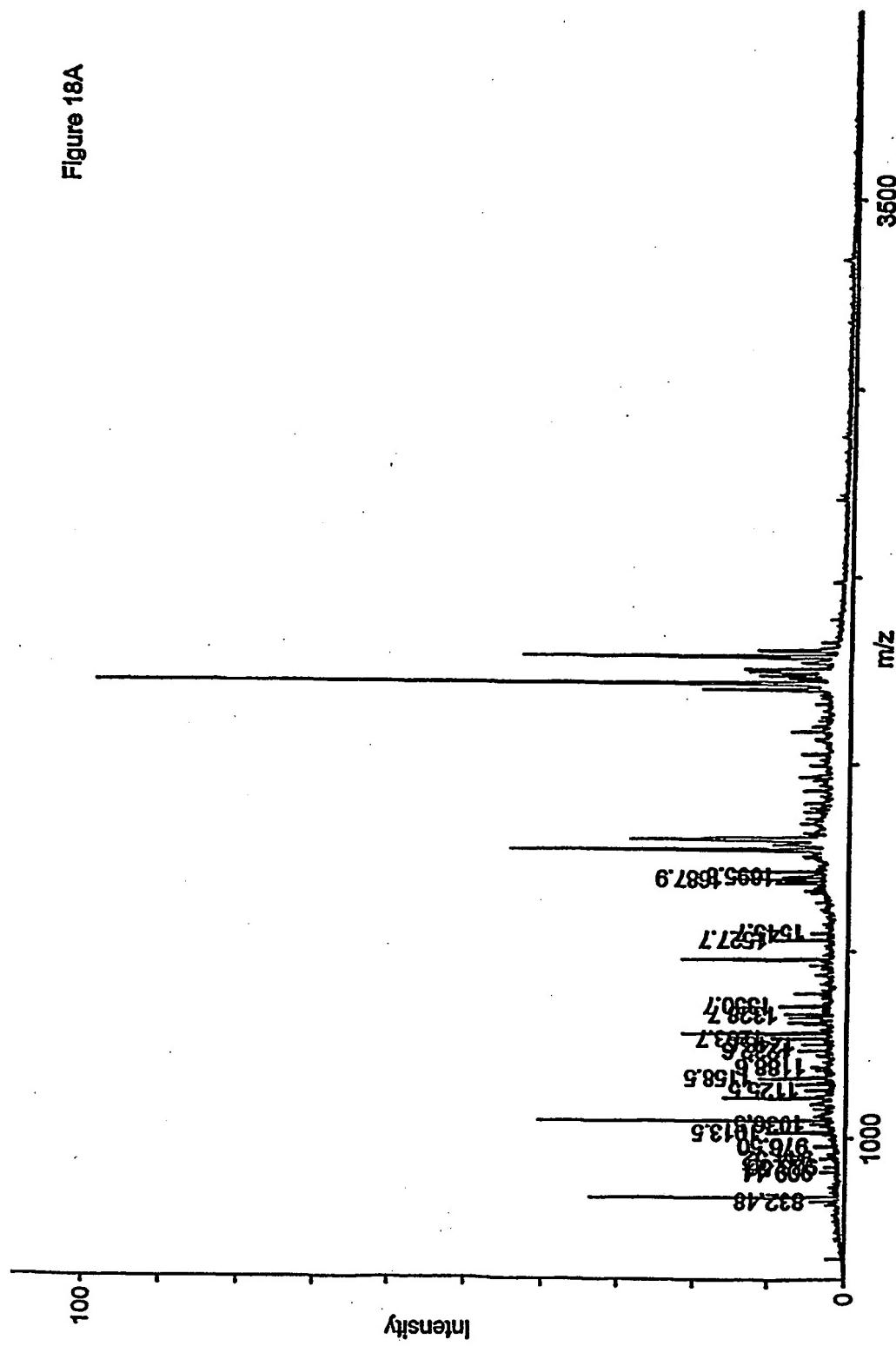
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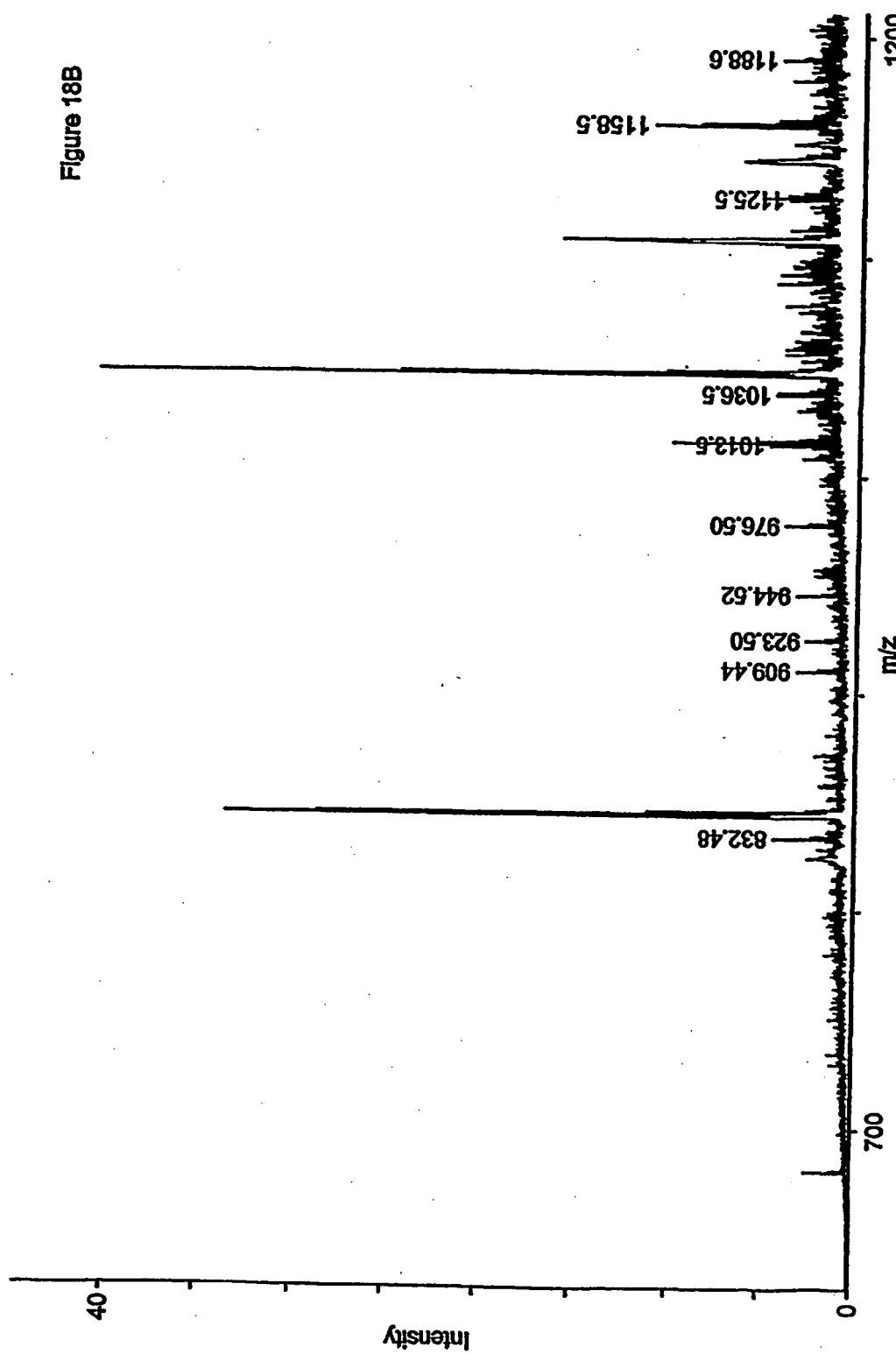
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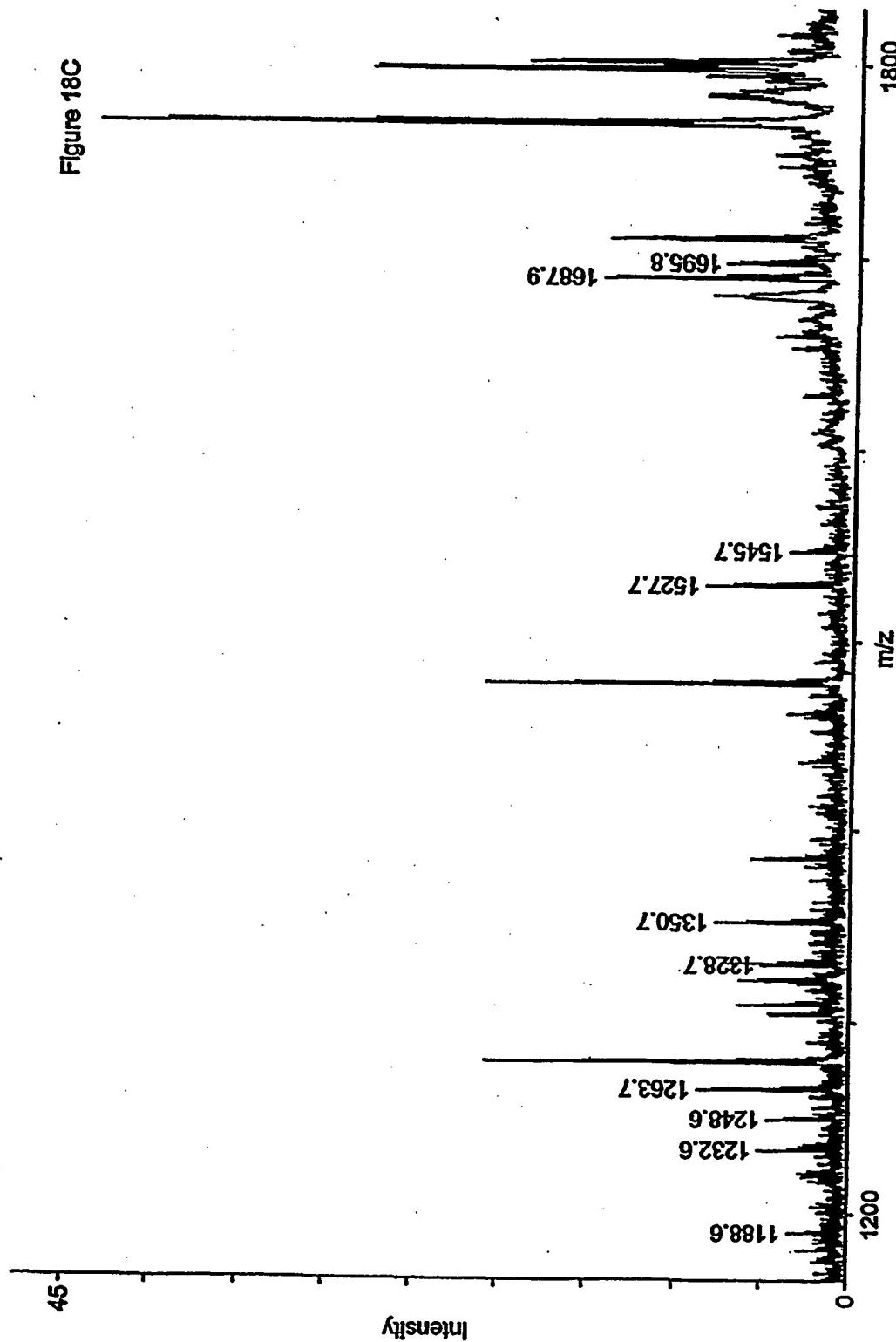
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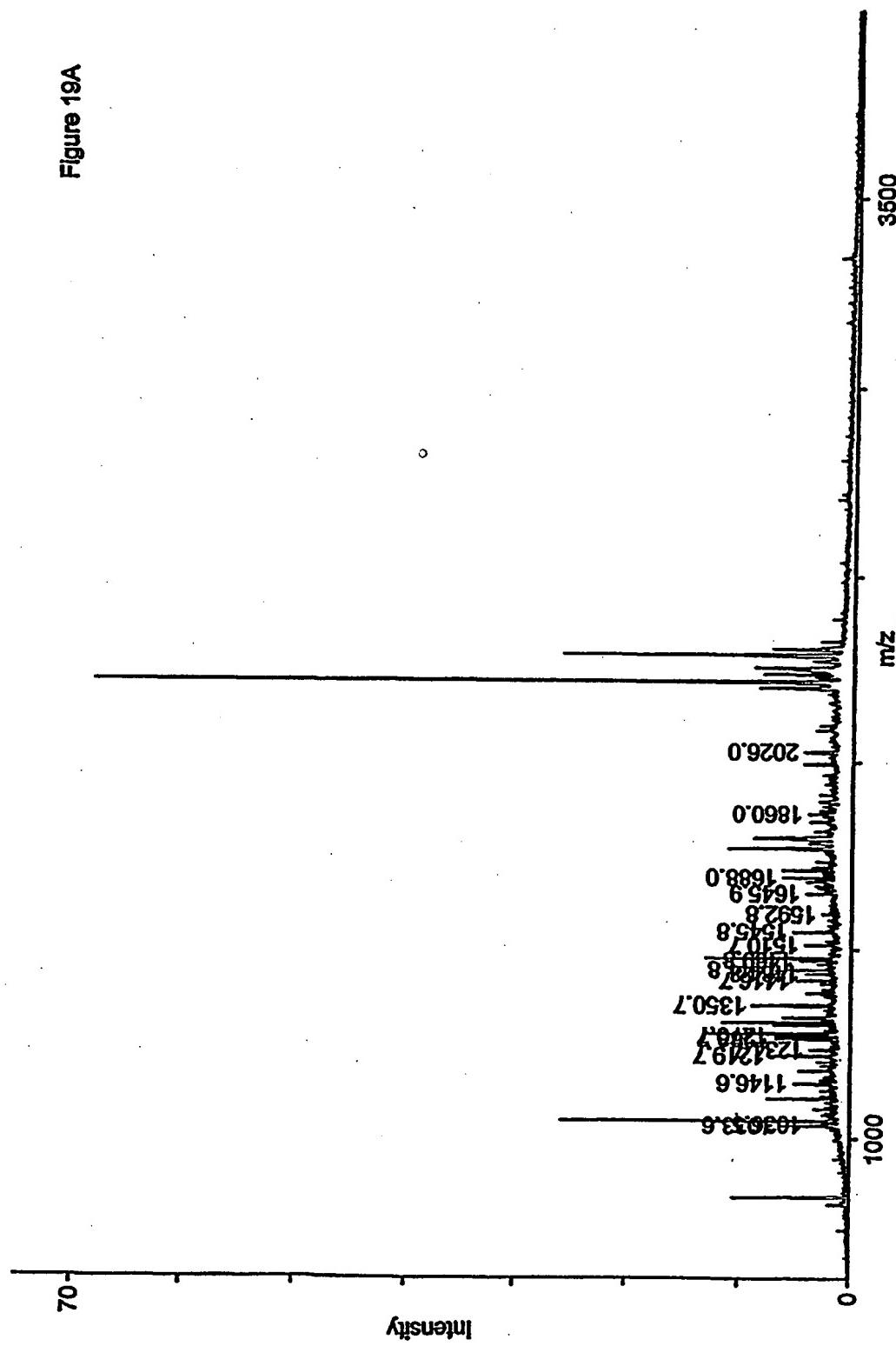
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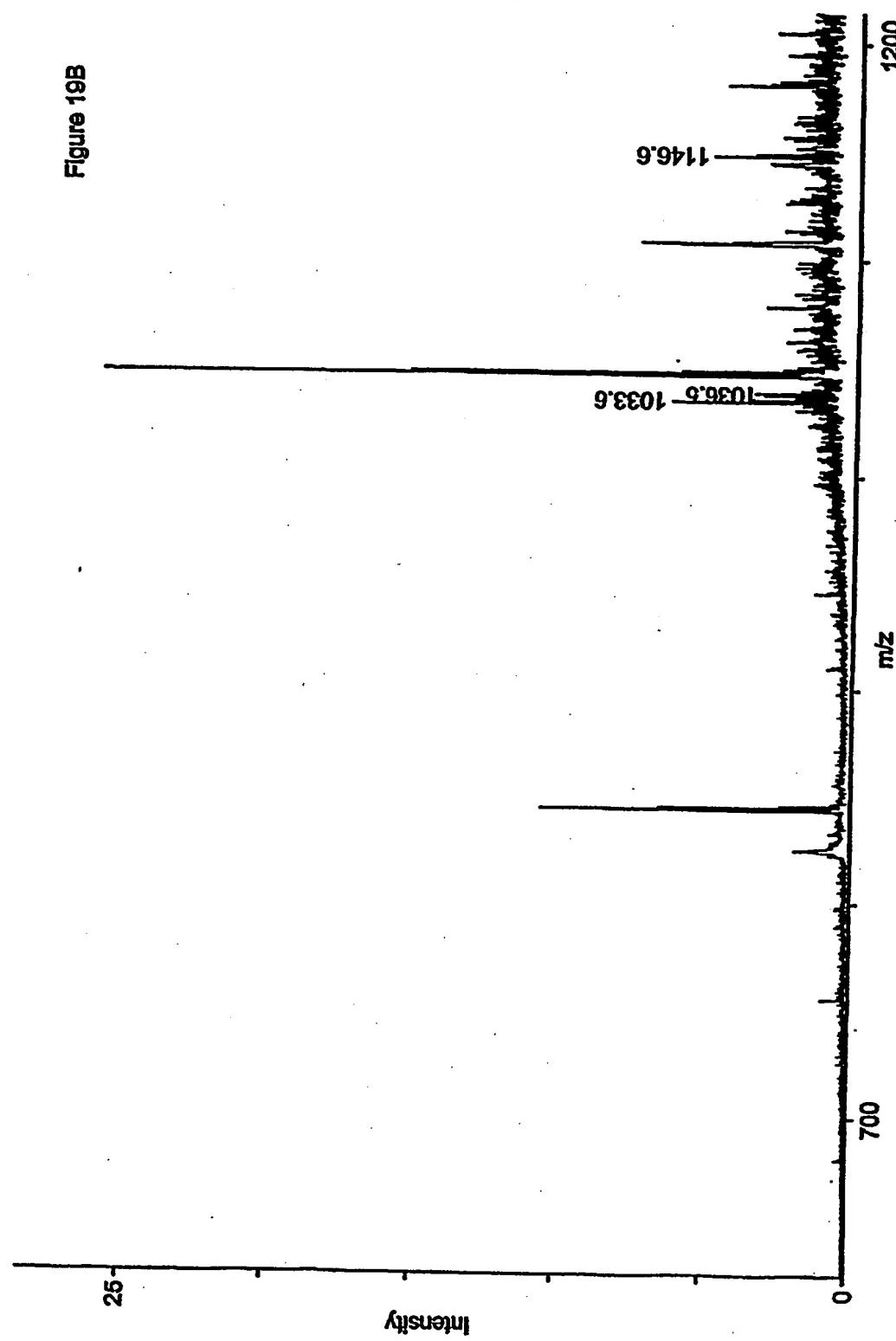
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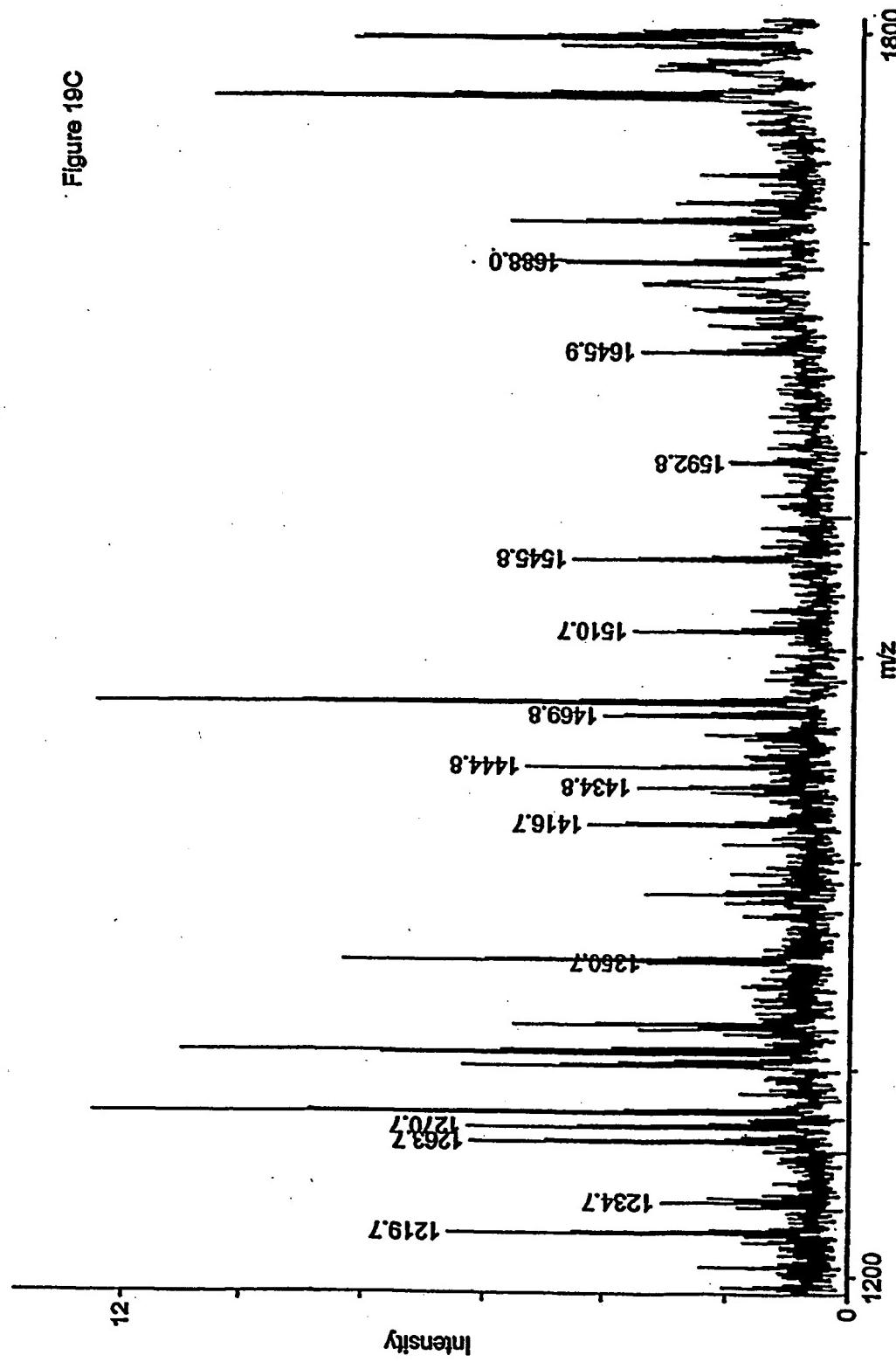
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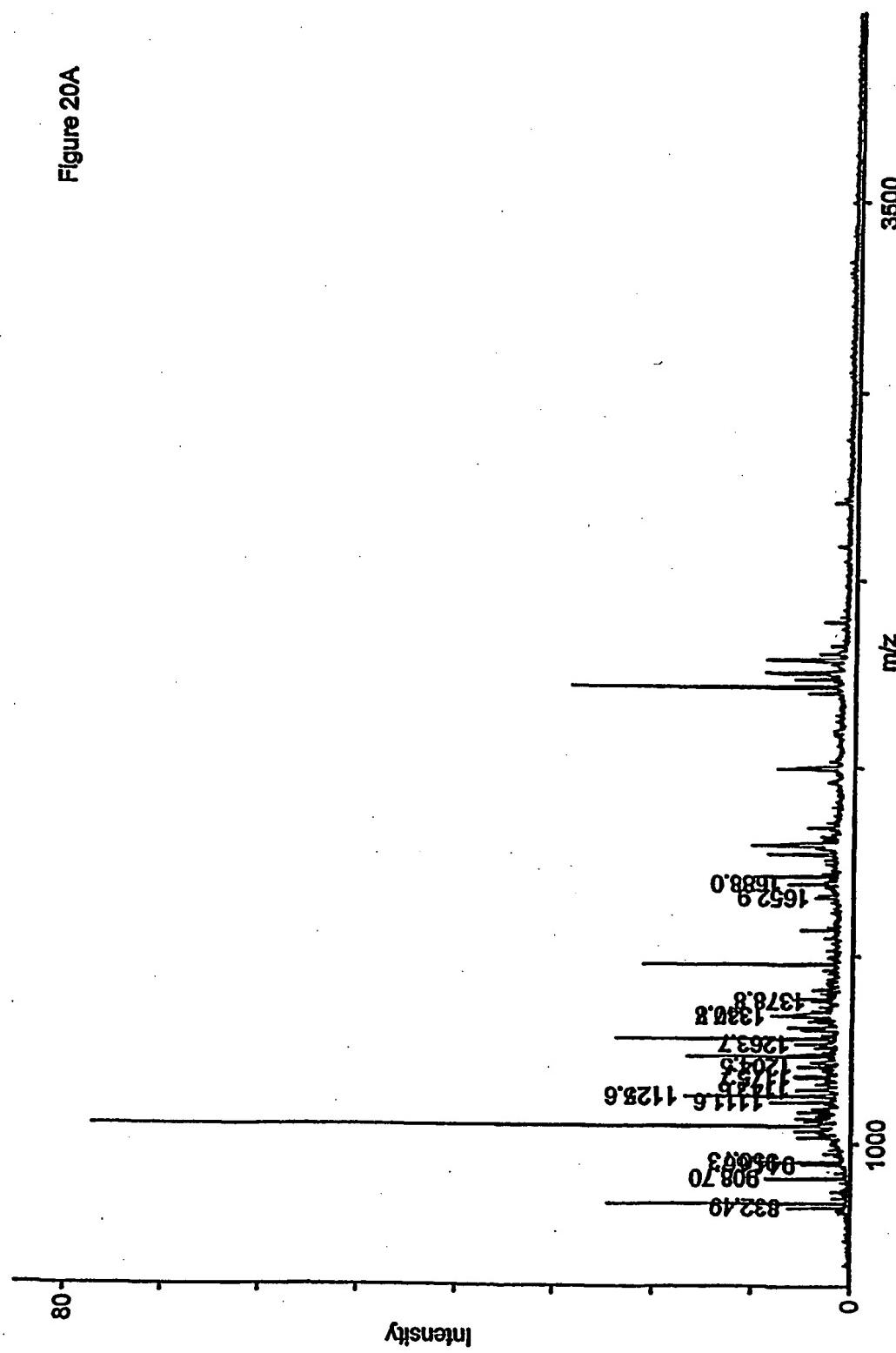
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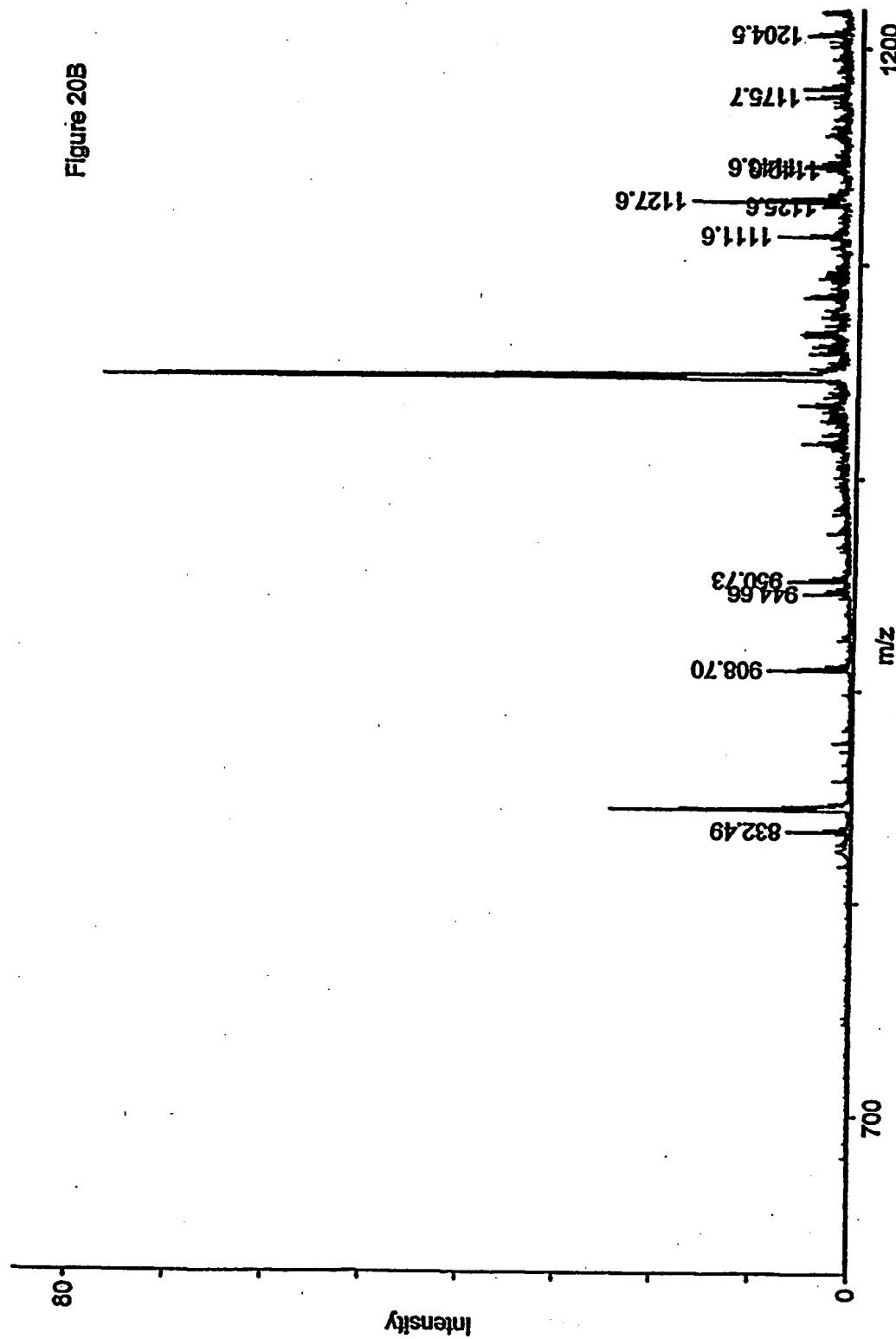
Figure 20C

Figure 21A

Figure 21B

Figure 21C

Figure 21D